EVIDENCE FOR THE FUNCTIONAL SIGNIFICANCE OF MICROBIAL DIVERSITY AMONG FREE-LIVING DIAZOTROPHS IN SOILS OF A LONG TERM AGRICULTURAL SITE

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Shifang Hsu

January 2011

EVIDENCE FOR THE FUNCTIONAL SIGNIFICANCE OF MICROBIAL DIVERSITY AMONG FREE-LIVING DIAZOTROPHS IN SOILS OF A LONG TERM AGRICULTURAL SITE

Shifang Hsu, Ph. D.

Cornell University 2011

Diazotrophs, microorganisms that fix atmospheric N_2 into NH_4, fill a crucial role in the nitrogen cycle providing the dominant natural source of fixed N in the biosphere. Model systems for N-fixation in soil are generally based on cultivated and symbiotic diazotrophs. However many of the diazotrophs in soils belong to groups that have yet to be cultivated in isolation. In soils, the non-cultivated free-living diazotrophs may play an important role for N fixation. In this project we sought to characterize the community structure of the free-living diazotrophs in soil and to determine whether changes in diazotroph community composition had functional consequences for N-fixation in soils. We examined diazotroph community, soil characteristics, and N-fixation rates over time in a long-term agricultural experiment located at the William H. Miner Institute in Chazy, Clinton County, New York. The results of this project show that agricultural management practices fundamentally altered the structure of the microbial community and that these changes in turn impacted the rate of N-fixation that was observed. The association between diazotroph diversity and N-fixation rates was not a simple function of richness but rather seemed influenced by short term temporal changes in community structure. N-fixation rates varied significantly over time and these changes were more strongly correlated to temporal variation in diazotroph community composition than to temporal variation in soil characteristics.

The results suggest that controls on N-fixation are dynamic and are responsive to the composition of the diazotrophic community. Since the microbial community is itself responsive to land management practices there results significant interactions between land management practices and temporal variation in N-fixation rates.

BIOGRAPHICAL SKETCH

Shifang Florence Hsu was born in Taipei, Taiwan in the hot summer. Shifang is the only child of Tseng-Bi Lung and Ming-Teh Hsu. She grew up in the full-developed urban area, but her mother often brought her to the coast or mountain parks for their family vacation (even though her father was always against these vacation ideas). When she was a little girl, she told her parents that her life goal was be a student forever. Her parents did not treat this as her daydream (although, it probably was). Instead, they told her that if she could really do it, they would support her in any way to help her to achieve this goal. Sometime during middle school, Shifang found her interesting in biology. Later, she turned to study social work and earned the BA in the same field in 1994. During social work training, Shifang realized she still wanted to study biology, so she chose to pursue Microbiology as her minor and fell in love with the complex microbial world. Shifang went on to earn an MS in Microbiology from Soochow University, Taipei, Taiwan in 1997. After graduation, she worked as a research assistant for two years then finally made her decision to seek a research career. In order to improve her English language skills and expand her research horizons, she took a chance to pursue a second MS in Molecular Biology from Montclair State University, NJ, U. S. A. In fall 2004, Shifang came to Cornell and joined a wonderful lab, which lead her to establish her research career in microbial ecology. Shifang has followed a somewhat non-traditional path in her research training and worked hard to keep on her path to a research career in microbial ecology. Shifang hopes her research career somehow can achieve her childhood dream to be a student forever… a student who always learns new things from microbial ecology through her life.

Think before you choose, but don’t regret…

ACKNOWLEDGMENTS

Without help, no one can complete the PhD program. Therefore, there are a number of people I want to acknowledge here.

First, all my committee members: Dr. Daniel Buckley, Dr. Esther Angert, Dr. Eugene Madsen, and Dr. Eric Nelson. I think I am one of lucky one's who had very supportive committee members; your critique and creative ideas stimulated my thinking and shared your knowledge with me. Many thanks to you.

Dan, I really appreciate you for taking me as your student. I want to put this statement here: "You are not just the best adviser I ever had, but you are the best adviser in the world". I learned so many things from you; the way to look at research, the way to manage lab founding, the way to take care of your students. I learned the positive attitude from you, not only for research but also for advising and for my career. The past two years have been kind of tough for me. If not for your help and understanding, I would not have been able to finish this work. I cannot thank you more.

My dear first labmates, Tyrrell and Varisa, we shared lot fun times in the lab and office. Jesse, Chris, Steve, Jude, James and Peter (of the boy's club), you taught me lot of "American culture", made me learn new "vocabularies", and tolerated my unclear English pronunciation; not to mention all of the good discussion and help for research. You were the best labmates. Kai, Tarah, Jijy and other my lab neighbors, thanks for sharing a good time with me. I also want to thanks the folks in the Department of Crop and Soil Sciences who have helped my research experience. Without your help, it would not have been easy to finish my research.

Let me take this chance to thank my family. My uncle Ming-Yi Hsu, aunt Louisa Tsao and aunt Nancy Ho, many many thanks for taking care of my parents and my grandma. My dear husband baby boy cousins Albert (Dada) Hsu and Jason (A-Ja) Hsu, thank you for allowing me to make fun of you when I got bored. Uncle Lung and aunt Dorris, cousin Anita, Jack and Ali, thanks so much for all your support and good company, so I didn't feel alone.

My friends from MAF, all of you, thanks for listening to my complaining, sharing happiness and sadness, getting together once a long while to watch videos. My first advisor Dr. Chao, thank for sharing good thoughts of research, and sorry I broke your record again. There are still many many more folks I need to thank. Thank all of you.

Last, I want to use this dissertation to acknowledge my parents. Their unconditional love and support are the reason I could accomplish whatever I wanted to do. They never pushed me to do things traditionally expected by society. This was not easy for them in our culture, but they protected me. The only two things they insist are that I should keep a healthy attitude for my life and don't ever regret any decisions. They only care for my health and give me their endless love. If I have made any achievement, it's because I have them, my parents, thank you, love you forever.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Overview of this dissertation

Dinitrogen fixation is a biological process carried out by bacterial and archaeal organisms known as diazotrophs. These organisms are important in environments where nitrogen limits primary production because they are the only organisms capable of converting N_2 to NH_4. Model systems for N-fixation in soil are generally based on cultivated and symbiotic diazotrophs. However many of the diazotrophs in soils belong to groups that have yet to be cultivated in isolation. In soils, the non-cultivated free-living diazotrophs may play an important role for N fixation. The contribution of these free-living diazotrophs to soil nitrogen fixation may have been neglected historically due to the difficulties associated with the cultivation of these organisms. The purpose of this dissertation is to characterize the community structure of the free-living diazotrophs in soil, to assess the benefit of these organisms to soil N fixation, and to understand the relationship between diazotrophic community composition and soil N-fixation.

Background

Importance of diazotrophs in soil

Microbial communities are a central element of the productivity and health of terrestrial ecosystems (Copley, 2000). They have board impacts on atmospheric chemistry and global climate by influencing budgets of gases, and also play an important role in biogeochemical nutrient cycles such as the nitrogen and carbon cycles. Although their ubiquity and importance to the biosphere are unquestionable, there are still substantial gaps in our understandings of soil microbial communities. A

major limitation has been the fact that the majority of soil microorganisms cannot be cultivated or characterized in laboratories. In addition, cultivable soil microorganisms may not demonstrate the same behaviors in the laboratory as they do in their own habitats (Amann *et al.*, 1995). In recent decades, molecular techniques have provided opportunities to examine soil microorganisms *in situ* without the need for cultivation. These types of techniques allow us to understand the ecological function of these mysterious microbes. The information obtained from *in situ* studies can provide information to enable the cultivation and characterization of these previously uncultivated microbes (Kirk *et al.*, 2004; Liles *et al.*, 2003).

Nearly 80% of the atmosphere is composed of N_2, but N_2 gas cannot be used by most organisms as a source of N. Nitrogen fixation is the process that coverts gaseous N_2 into ammonium which is then available for other of forms of life. Biological N-Fixation (BNF) is the major natural and fundamental process through which fixed nitrogen is made available to the biosphere and this process can only be carried out by diazotrophic species of Bacteria and Archaea (Vitousek *et al.*, 2002). Free-living diazotrophs are estimated to fix 100-290 Tg of N per year in terrestrial systems (Cleveland *et al.*, 1999).

The use of molecular techniques for studying non-cultivated N-fixing microorganisms has identified massive numbers of non-cultivated diazotrophs in environmental samples. Currently there are more than 12,000 sequences of nitrogenase *nifH* genes in GenBank. These *nifH* genes can be grouped into approximately 50 clusters; half of which contain no cultivated isolate(Zehr *et al.*, 2003). Even within groups that contain a cultivated isolate most sequences are not closely related to a characterized diazotroph (Ueda *et al.*, 1995; Zehr *et al.*, 2003). Nitrogenase gene sequences reveal numerous non-cultivated diazotrophs in terrestrial systems, but the functional significance these free-living non-cultivated diazotrophs

remains uncharacterized. More research is needed in order to understand the importance of free-living non-cultivated diazotroph in terrestrial systems.

Non-cultivated diazotrophs in soils: limit of our knowledge

Many studies have been conducted for understanding the diazotrophic community in marine systems as BNF is the major N input in these systems, especially in the open ocean (Zehr *et al.*, 1998). Studies of BNF in terrestrial systems, however, have mostly focused on symbiotic diazotrophs due to their agronomic importance. Major crops such as soybean, alfalfa, peas, clover, and beans obtain substantial amounts of their nitrogen from the N-fixation of their symbiotic diazotrophs. These symbiotic bacteria are also generally easy to isolate from the environment and to handle in laboratory experiments (Vergin *et al.*, 1998). As a result most of our knowledge of N-fixation in terrestrial systems is focused on symbiotic N-fixation and less knowledge is available about N-fixation by free-living organisms in soils.

The basis for understanding free-living diazotrophs in soil has been derived mostly from studies conducted with cultivated isolates; however, these organisms only represent a small minority of actual diazotrophic communities (Hamelin *et al.*, 2002; Poly *et al.*, 2001). The free-living N-fixing organisms that have been isolated from the soil and studied in pure culture (i.e.: *Azotobactor, Azospirillum, Klebsiella, Clostridum, Azoacrus, Azotrhizobium*) are known to be overrepresented in culture collections relative to their actual abundance in soil (Elmerich *et al.*, 1987; Holguin *et al.*, 1999; Leigh, 2002; Newton, 2004; Postgate, 1998b). Because it is a lot easier to measure the N-fixation in the symbiotic diazotrophic system than free-living, particularly in the agricultural system, it is not clear that the degree to which studies of N-fixation by cultivated strains in balanced growth conditions are representative of the

constraints that govern N-fixation by not yet cultivated strains *in situ* in the soil (Kennedy and Islam, 2001). Therefore, the contributions of BNF from free-living non-cultivated diazotrophs may have been historically underestimated.

The Acetylene Reduction Assay and issues of measuring N-fixation in situ

The Acetylene Reduction Assay (ARA) was developed in the late 1960's (Hardy *et al.*, 1968) and has become a standard method for BNF measurements. Prior to the development of ARA the best method for measuring N-fixation was through the use of $^{15}N_2$, however, the method was difficult to apply in the mid-twentieth century because the expense of working with stable isotopes was still largely prohibitive. In the laboratory, ARA is easy to handle, repeatable inexpensive, and generally reliable for measuring N-fixation of symbiotic BNF. The basis of ARA is the ability of nitrogenase to react with acetylene (C_2H_2) in place of dinitrogen, reducing it to ethylene (C_2H_4). The production of ethylene can then be rapidly and sensitively measured by gas chromatography using a flame ionization detector. The conversion of acetylene to ethylene requires 2 electrons while fixation of dinitrogen requires a minimum of 6 electrons. Hydrogen gas is also produced by nitrogenase as a consequence of N-fixation and thus the actual number of electrons required for N-fixation can vary. Three types of nitrogenase, which are over through most known N-fixation enzymes, have been well characterized their enzyme activities. These nitrogenases were categorized by co-factors of active site: molybdenum-dependent, vanadium-dependent and iron-dependent. The molybdenum (Mo) dependent nitrogenase is generally considered to require 8 electrons per molecule of dinitrogen reduced, though, in strains with uptake hydrogenase activity the electrons 'wasted' in hydrogen production can be recycled. Thus, application of ARA requires an assumption of the ratio of C_2H_2 reduction: N_2 reduction, and the most commonly used

conversion ratios are 3 (Galloway *et al.*, 2004) and 4 (Postgate, 1968). It is important to consider that Vanadium (V) - dependent nitrogenases may be less efficient than Mo-dependent nitrogenases, producing more hydrogen, and requiring greater numbers of electrons per molecule of dinitrogen reduced (Eady *et al.*, 1987; Haaker and Klugkist, 1987). In additional, V- dependent nitrogenases can therefore generate C_2H_6 instead of C_2H_4 from acetylene (Dilworth *et al.*, 1987). Given these factors that can influence the ARA conversion ratio, best practice still requires calibration of the C_2H_2:N_2 reduction ratio by using $^{15}N_2$ reduction into cell biomass as a standard for each strain under investigation (Postgate, 1968).

ARA is widely used to estimate N-fixation rates *in situ*, however, such application is fraught with biases that can be difficult to constrain, particularly in soils. Application *in situ*, as above, must assume a C_2H_2:N_2 reduction ratio. Although comparisons between ARA and $^{15}N_2$ fixation rates in many ecosystems have shown good agreement with theoretical reduction ratios, this is not always the case and the actual ratio can vary (Liengen, 1999; Postgate, 1982). Calibration of the C_2H_2 reduction assay has been recommended in each system considered (Postgate, 1982). A problem arises in that changes in community structure or relative N-fixation activity by different organisms over time might reasonably be expected to alter the actual conversion ratio requiring frequent re-calibration. Acetylene is also a known inhibitor for many microbial activities in soil. Some microorganisms inhibited by acetylene have been also found to have the ability to perform nitrogen fixation (Gadkari *et al.*, 1992; Postgate, 1998a). Finally, the diffusion coefficients and solubility of dinitrogen, ethylene, and acetylene can vary in different solutions. In bulk soil systems this can be an important consideration since factors that limit the diffusion of acetylene relative to dinitrogen will cause underestimation of N-fixation rates by ARA. Microbes and

plants can also produce or consume ethylene further complicating application of ARA in soil systems(Minchin *et al.*, 1986; Minchin *et al.*, 1994).

The major issues associated with using ARA to measure *in situ* activities were addressed further by Montoya and colleagues (Montoya *et al.*, 1996). They compared the ARA for measuring N-fixation in ocean samples with the method of directly measuring the changes in $\delta^{15}N$ abundance in ocean samples incubated with $^{15}N_2$ gas. Their results showed that ARA systematically underestimated actual N-fixation rates relative to the level of $\delta^{15}N$ enrichment observed. In addition, conversion factors in the range of 0.022 to 22 have been reported for terrestrial habitats in which ARA has been standardized with $^{15}N_2$ based measurements (Nohrstedt, 1983; Skujins *et al.*, 1987; Spiff, 1973; Zechmeister-Boltenstern and Kinzel, 1990). These results suggested that ARA could underestimate actual N-fixation rates by more than two orders of magnitude.

Stable isotope measurement of N-fixation in soil

It is possible that reliance on ARA over the last 40 years has led to systematic mis-estimation of the role of free-living diazotrophs in many soils. While still more expensive and cumbersome than the ARA, decreases in the expense of working with stable isotopes now means that $^{15}N_2$ based measurements can be feasibly applied to field level studies of N-fixation. There are two different approaches by which stable isotopes can be used to estimate N-fixation rates. The first is through measurement of ^{15}N natural abundance and the second is through enrichment with $^{15}N_2$. The ^{15}N isotope occurs naturally and represents 0.36 % of the N_2 in the atmosphere with the balance $^{14}N_2$. Many processes cause isotopic fractionation of ^{15}N leading soils and biomass to generally have $\delta^{15}N$ values that differ significantly from the atmospheric N_2 reservoir. Nitrogenase, however, has almost no isotopic fractionation and thus, N-

fixation will cause δ ^{15}N values to move towards atmospheric levels of enrichment. This means that if a control can be identified in which N-fixation is not occurring it is possible to estimate N-fixation in samples where N-fixation is active. This method is most commonly used to estimate N-fixation rates in legumes where it is possible to find cultivars which are deficient in nodule formation. The need for non-N_2 fixing controls and/or reference samples limits the utility of this method. The second approach involves addition of $^{15}N_2$ and measurement of sample ^{15}N enrichment by mass spectrometry. Since chemical reduction of N_2 does not occur under standard conditions and fractionation by nitrogenase is negligible, estimation of N-fixation rates is a simple matter of comparing ^{15}N enrichment in samples relative to controls that do not receive $^{15}N_2$. This method has been widely applied (Brouzes *et al.*, 1969; Magurran, 1988; Montoya *et al.*, 1996; Nohrstedt, 1983; O'Toole and Knowles, 1973; Skujins *et al.*, 1987) and has traditionally been recommended to verify N-fixation rates in cases where ARA is used(Stewart *et al.*, 1967).

We evaluated this method for its applicability in estimating N-fixation in soils. A major consideration is the need for appropriate controls. $^{15}N_2$-fixation leads to stabilization of ^{15}N into soil organic matter. Organic nitrogen can subsequently be mineralized which can lead to ^{15}N-fractionation, but will not impact the bulk ^{15}N signature of soil unless N is lost from the system. Gaseous N loss caused by fractionating processes, such as nitrification, denitrification, and ammonia volatilization, can impact the ^{15}N-enrichment of bulk soil, thus, it is necessary to control for these processes. Leaching can also alter the bulk ^{15}N-enrichment of soil but this process can be neglected when assays are conducted in sealed containers. To control for the possibility of gaseous N losses and their impact on ^{15}N pools it is necessary to incubate all samples that receive $^{15}N_2$ in parallel with controls that instead receive $^{14}N_2$. In the method that we developed, 5 g soil was placed into 18 × 150 mm

Balch tubes (Bellco Glass, Vineland, New Jersey), and the headspace was replaced with synthetic air containing 20% O_2 and 80% $^{15}N_2$ (>98 atom % ^{15}N, Isotec, Miamisburg, Ohio). Controls were processed in parallel and received unlabeled N_2 gas. Tubes were incubated horizontally at room temperature in the dark for 9 days. The atom % ^{15}N of soil samples was determined using a Finnigan MAT Delta Plus mass spectrometer (Thermo Electron Corporation, Waltham, Massachusetts) plumbed to a Carlo Erba NC2500 elemental analyzer (CE Instruments, Wigan, UK) through a Conflo II open split interface for elemental and isotopic composition of solid samples (Thermo Electron Corporation). The net potential N-fixation rate was calculated from the difference of total ^{15}N in soils receiving $^{15}N_2$ relative to controls. It should be recognized that potential rates can differ from *in situ* rates due to bottle effects, but such potential rates are commonly useful for assessing relative differences in the activities of microbial communities.

We used the $^{15}N_2$-enrichment method described above as our sole method for measuring potential N-fixation rates. Several tests were performed in soils from 2 different sites to confirm that N-fixation rates determined with this method would respond appropriately to treatments known to impact N-fixation rates (see appendix A). First, either autoclaving soil, adding 300 μg/g ammonium nitrate, or adding 40% acetylene (a competitive inhibitor of N-fixation) were all observed to reduce N-fixation rates relative to controls while addition of 25 mg/g glucose was observed to increase the N-fixation rate and these differences were all significant (Appendix A). Addition of 25 mg/g ammonium sulfate reduced the N-fixation rates relative to control, but this difference was not significant. The stimulation N-fixation that occurred due to glucose addition was eliminated when glucose was added with of 25 mg/g ammonium sulfate (Appendix A).

C and N availability and free-living diazotrophs in soil

A range of soil parameters can potentially impact N-fixation rates by free-living diazotrophs. Previous studies have suggested that the free-living diazotrophs in soil are generally C and energy limited. The impact of C resources on N-fixation varies in different ecosystems (Vitousek *et al.*, 2002). When supplemented with glucose, a compound used by most cultivated N-fixers, N-fixation can be stimulated in certain soils (Kondo, 2003) but in other cases the glucose provides inconsistent results or no stimulation at all (Roper and Smith, 1991). Similar results occur when cellulose was added (Kondo, 2003). Piceno *et al* (Piceno and Lovell, 2000b) suggested that changes in C availability from root exudates had no effect on either N-fixation rate or *nifH* community composition. These results suggested that C availability is not enough to explain low rates of N-fixation in some systems. The different responses of diazotrophs to substrate enrichment may be a result of either differences in soil characteristics (Roper and Smith, 1991), or differences in diazotrophic community composition, or both.

The influence of bulk soil N availability on N-fixation is unclear. Chemical fertilizers have been observed to decrease N-fixation in rice roots in certain soils (Tal and Okon, 1985), but not in others (Bagwell and Lovell, 2000; Blackwood *et al.*, 2003; Tal and Okon, 1985). The effect of N availability on the diazotrophic community is also unclear. Using *nifH* to monitor diazotrophic communities in upland soil or salt marsh soil showed no relationship between the diazotroph community composition and N availability (Piceno and Lovell, 2000a; Poly *et al.*, 2001). Though soil C and N availability should be expected to impact N-fixation and diazotrophic community composition, the uncertain results of these studies did not clarify the roles that free-living diazotrophs play in N-fixation of the soils. There may be other factors

beside C and N that can also influence diazotrophic activity. More studies need to be done in order to understand the roles of diazotrophs in soil.

Free-living N-fixation in Agroecosystems

Non-symbiotic N-fixation is the major resource for N input in many natural systems (Cleveland *et al.*, 1999), but the potential importance of free-living diazotrophic N-fixation to cropping systems is debatable. A theoretical limitation to the contribution of free-living diazotrophs to agroecosystems is that these organisms fix N_2 gas to satisfy their own N needs; which is unlike the symbionts that produce N mainly for their hosts. However, N contained in biomass is also available for crops as a result of biomass cycling (Lethbridge and Davidson, 1983). Management decisions to increase soil-N fixation from free-living diazotrophs may result in longer term increases in the soil N which is ultimately made available to plans through subsequent mineralization.

Two agricultural practices that can encourage the activity of free-living diazotrophs are no-till systems and straw retention. In no-till systems, soil structure is maintained preserving soil aggregates where N-fixation may be highly favored (Chotte *et al.*, 2002). The use of plant residues with high C:N ratio biomass contants have long been known for stimulating nitrogenase activity in the field (Roper *et al.*, 1994). Kennedy and Islam (Kennedy and Islam, 2001) calculated that the complete retention of straw from a wheat crop could potentially yield 10-150 Kg N per ha. The potential impacts of free-living diazotrophs on N-fixation in agricultural systems remain incompletely characterized. Agricultural activities have been shown to influence the community structure and activity of other functional groups in soil such as denitrifiers (Cavigelli and Robertson, 2000; Rich and Myrold, 2004). In addition, the diversity of free-living diazotrophs has been shown to impact N-fixation in marine ecosystems

(Montoya *et al.*, 2007), but the functional significance of diazotroph community in soil systems still remains largely uncharacterized.

Available tools for studying functions of free-living diazotrophs in soil

Molecular techniques can be used to study free-living diazotrophs in soil, but linking the identity and activity of microorganisms *in situ* remains a challenge. Certain molecular techniques can be used to study gene expression in microorganisms in soil environments. This can provide opportunities to link community composition and function in terrestrial systems (Saleh-Lakha *et al.*, 2005). Analysis of mRNA from soil samples can be subjected by studying gene expression under specific conditions. Nitrogenase mRNA has been used for studying the diazotrophic activity to monitor the effects of root exudates on structure and activity of a soil diazotroph community. Burgmann and colleagues used nitrogenase *nifH* mRNA to demonstrate the functional nitrogen-fixing population only represented a fraction of the total diazotroph diversity, and substrate concentration may have additional selective effects on the active diazotroph population (Burgmann *et al.*, 2007). Real-Time PCR has been used for quantifying the presence of certain functional genes in environmental samples and has successfully been used for quantification of the abundance of denitrifying bacteria (Gruntzig *et al.*, 2001). Therefore, Real-Time PCR can be applied to quantify abundance of diazotrophic populations with nitrogenase genes in soil.

Research objectives

The primary objective of this dissertation was to understand how specific agricultural management practices influence the diversity and metabolic function of free-living diazotrophs in soil. The goal was to use a well-characterized long-term

agricultural site which represents a gradient of soil characteristics to determine the ecological significance of free-living diazotrophs in soils and to reveal the principles that govern their fitness and activity in soil systems. To achieve these purposes we focused on a long-term agricultural experiment site in Chazy, Clinton, NY (described in Chapter 2). The site consists of a 2 x 2 factorial design testing the effects of tillage and biomass retention practices without crop rotations. These treatments have generated a gradient of organic matter and C availability at the site which could impact the structure and function of the diazotrophic community. The Chazy site was established in 1973 allowing the system to equilibrate for >30 years. This is an important consideration since microbial communities in soil have been shown to take long periods of time to respond to changes in land management practice (Buckley and Schmidt, 2001; Buckley and Schmidt, 2003). We conducted sampling in 2005 and 2006 using analysis of *nifH* gene sequences and *nifH* TRFLP to examine the structure of the diazotrophic community in relation to measurements of soil characteristics and N-fixation rates. In addition, experiments were conducted with N-free media and soil microcosms to explore the mechanisms by which diazotroph community composition might impact N-fixation rates. The focus of each chapter is described below:

Chapter 2 focuses on samples from November 2005 to examine the effects of tillage and biomass management on diazotroph diversity, soil characteristics, and soil N-fixation rates. The data that we describe suggest that differences in diazotroph community structure influence rates of N-fixation at the Chazy site.

Chapter 3 reports the relationship between N-fixation rate, soil characteristics, and diazotrophic community structure in soil over time. *nifH* TRFLP was used to analyze the composition of the soil diazotrophic community under different agricultural treatments and times. A general linger regression (GLM) model is used to

evaluate the impacts of diazotrophic community, soil characteristics, treatment, and time on N-fixation rates.

Chapter 4 explores factors that impact diazotrophic community structure at different agricultural management sites. In this chapter we contrast the Chazy site with a separate long-term agricultural management site Caldwell field in Ithaca, Tompkins, NY. Caldwell was also established more than 35 yrs ago and has been under continuous maize cultivation though under different tillage and biomass management routines from Chazy. The chapter reveals distinct diazotrophic groups that are found only in association with the maize fields and others that are unique to never cultivated sites.

Chapter 5 explores the hypothesis that the impact of diazotroph diversity on N-fixation rates is mediated through functional diversity and functional redundancy. Both cultivation dependent and microcosm experiments were used and though the data is not conclusive it is consistent with the hypothesis proposed.

Chapter 6 provides a brief conclusion to the dissertation.

REFERENCES

Amann RI, Ludwig W, Schleifer KH (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59:** 143-169.

Bagwell CE, Lovell CR (2000). Microdiversity of Culturable Diazotrophs from the Rhizoplanes of the Salt Marsh Grasses Spartina alterniflora and Juncus roemerianus. *Microbial Ecology* **39:** 128-136.

Blackwood CB, Marsh T, Kim S-H, Paul EA (2003). Terminal Restriction Fragment Length Polymorphism Data Analysis for Quantitative Comparison of Microbial Communities. *Appl. Environ. Microbiol.* **69:** 926-932.

Brouzes R, Lasik J, Knowles R (1969). Effect of Organic Amendment, Water Content, and Oxygen on Incorporation of $15N_2$ by Some Agricultural and Forest Soils. *Can J Microbiol* **15:** 899-905.

Buckley D, Schmidt T (2001). The structure of microbial communities in soil and the lasting impact of cultivation. *Microbial Ecology* **42:** 11-21.

Buckley DH, Schmidt TM (2003). Diversity and dynamics of microbial communities in soils from agro-ecosystems. *Environmental Microbiology* **5:** 441-452.

Burgmann H, Howard EC, Ye W, Sun F, Sun S, Napierala S *et al* (2007). Transcriptional response of Silicibacter pomeroyi DSS-3 to dimethylsulfoniopropionate (DMSP). *Environmental Microbiology* **9:** 2742-2755.

Cavigelli MA, Robertson GP (2000). The Functional Significance Of Denitrifier Community Composition In A Terrestrial Ecosystem. *Ecology* **81:** 1402-1414.

Chotte J-L, Schwartzmann A, Bally R, Jocteur Monrozier L (2002). Changes in bacterial communities and Azospirillum diversity in soil fractions of a tropical soil under 3 or 19 years of natural fallow. *Soil Biology and Biochemistry* **34:** 1083-1092.

Cleveland CC, Townsend AR, Schimel DS, Fisher H, Howarth RW, Hedin LO *et al* (1999). Global patterns of terrestrial biological nitrogen (N2) fixation in natural ecosystems. *Global Biogeochem. Cycles* **13:** 623-645.

Copley J (2000). Ecology goes underground. *Nature* **406:** 452-454.

Dilworth MJ, Eady RR, Robson RL, Miller RW (1987). Ethan formation from acetylene as a potential test for vanadium nitrogenase in vivo. *Nature* **327:** 167-168.

Eady RR, Robson RL, Richardson TH, Miller RW, Hawkins M (1987). The vanadium nitrogenase of Azotobacter chroococcum. *Biochem. J.* **244:** 197-207.

Elmerich C, Bozouklian H, Vieille C, Fogher C, Perroud B, Perrin A *et al* (1987). Azospirillum: Genetics of Nitrogen Fixation and Interaction with Plants [and Discussion]. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **317:** 183-192.

Gadkari D, Morsdorf G, Meyer O (1992). Chemolithoautotrophic assimilation of dinitrogen by Streptomyces thermoautotrophicus UBT1: identification of an unusual N2-fixing system. *J. Bacteriol.* **174:** 6840-6843.

Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP *et al* (2004). Nitrogen Cycles: Past, Present, and Future. *Biogeochemistry* **70:** 153-226.

Gruntzig V, Nold SC, Zhou J, Tiedje JM (2001). Pseudomonas stutzeri Nitrite Reductase Gene Abundance in Environmental Samples Measured by Real-Time PCR. *Appl. Environ. Microbiol.* **67:** 760-768.

Haaker H, K|ugkist J (1987). The bioenergetics of electron transport to nitrogenase. *FEMS Microbiology Reviews* **46:** 57-71.

Hamelin J, Fromin N, Tarnawski S, Teyssier-Cuvelle S, Aragno M (2002). *nifH* gene diversity in the bacterial community associated with the rhizosphere of Molinia coerulea , an oligonitrophilic perennial grass. *Environmental Microbiology* **4:** 477-481.

Hardy RWF, Holsten RD, Jackson EK, Burns RC (1968). The Acetylene-Ethylene Assay for N_2 Fixation: Laboratory and Field Evaluation. *Plant Physiol.* **43:** 1185-1207.

Holguin G, Patten CL, Glick BR (1999). Genetics and molecular biology of Azospirillum. *Biology and Fertility of Soils* **29:** 10-23.

Kennedy IR, Islam N (2001). The current and potential contribution of asymbiotic nitrogen fixation to nitrogen requirements on farms: a review. *Australian Journal of Experimental Agriculture* **41:** 447-457.

Kirk JL, Beaudette LA, Hart M, Moutoglis P, Klironomos JN, Lee H *et al* (2004). Methods of studying soil microbial diversity. *Journal of Microbiological Methods* **58:** 169-188.

Kondo MaY, Michio (2003). Effects of Temperature, Water Regime, Light, and Soil Properties on $15N_2$ Fixation Associated with Decomposition of Organic Matter in Paddy Soils. *Japan Agricultural Research Quarterly* **VOL.37:** 113-119.

Leigh GJ (2002). *Nitrogen Fixation at the Millennium* Elsevier B.V.

Lethbridge G, Davidson MS (1983). Microbial biomass as a source of nitrogen for cereals. *Soil Biology and Biochemistry* **15:** 375-376.

Liengen T (1999). Conversion factor between acetylene reduction and nitrogen fixation in free-living cyanobacteria from high arctic habitats. *Can J Microbiol* **45:** 223-229.

Liles MR, Manske BF, Bintrim SB, Handelsman J, Goodman RM (2003). A Census of rRNA Genes and Linked Genomic Sequences within a Soil Metagenomic Library. *Appl. Environ. Microbiol.* **69:** 2684-2691.

Magurran AE. (1988). International Society for Microbial Ecology.

Minchin FR, Sheehy JE, Witty JF (1986). Further Errors in the Acetylene Reduction Assay: Effects of Plant Disturbance. *Journal of Experimental Botany* **37:** 1581-1591.

Minchin FR, Witty JF, Mytton LR (1994). Reply to 'Measurement of nitrogenase activity in legume root nodules: In defense of the acetylene reduction assay' by J.K. Vessey. *Plant and Soil* **158:** 163-167.

Montoya JP, Voss M, Capone DG (2007). Spatial variation in N_2-fixation rate and diazotroph activity in the Tropical Atlantic. *Biogeosciences* **4:** 369-376.

Montoya JP, Voss M, Kahler P, Capone DG (1996). A Simple, High-Precision, High-Sensitivity Tracer Assay for N_2 Fixation. *Appl. Environ. Microbiol.* **62:** 986-993.

Newton WE (2004). *Genetics and Regulation of Nitrogen Fixation in Free-Living Bacteria*. Springer.

Nohrstedt HO (1983). Conversion factor between acetylene reduction and nitrogen fixation in soil: effects of water content and nitrogenase activity. *Soil Biol Biochem* **15:** 275-279.

O'Toole P, Knowles R (1973). Efficiency of acetylene reduction (nitrogen fixation) in soil: Effect of type and concentration of available carbohydrate. *Soil Biology and Biochemistry* **5:** 789-797.

Piceno YM, Lovell CR (2000a). Stability in Natural Bacterial Communities: I. Nutrient Addition Effects on Rhizosphere Diazotroph Assemblage Composition. *Microbial Ecology* **39:** 32-40.

Piceno YM, Lovell CR (2000b). Stability in Natural Bacterial Communities: II. Plant Resource Allocation Effects on Rhizosphere Diazotroph Assemblage Composition. *Microbial Ecology* **39:** 41-48.

Poly F, Ranjard L, Nazaret S, Gourbiere F, Monrozier LJ (2001). Comparison of *nifH* Gene Pools in Soils and Soil Microenvironments with Contrasting Properties. *Appl. Environ. Microbiol.* **67:** 2255-2262.

Postgate J (1998a). *Nitrogen Fixation, 3rd Edition*, third edn. Cambridge University Press , Cambridge, UK.

Postgate JR (1968). *Nitrogen Fixation, , 3rd Edition*, 3rd ed edn. The Press Syndicate of the University of Cambridge, Cambridge, UK.

Postgate JR (1982). *The fundamentals of nitrogen fixation*. Cambridge Univ. Press: Cambridge (UK), 252pp.
Postgate JR (1998b). *Nitrogen Fxation*. Cambridge: Cambridge University Press.

Rich JJ, Myrold DD (2004). Community composition and activities of denitrifying bacteria from adjacent agricultural soil, riparian soil, and creek sediment in Oregon, USA. *Soil Biology and Biochemistry* **36:** 1431-1441.

Roper MM, Smith NA (1991). Straw decomposition and nitrogenase activity (C_2H_2 reduction) by free-living microorganisms from soil: Effects of pH and clay content. *Soil Biology and Biochemistry* **23:** 275-283.

Roper MM, Turpin JE, Thompson JP (1994). Nitrogenase activity (C_2H_2 reduction) by free-living bacteria in soil in a long-term tillage and stubble management experiment on a vertisol. *Soil Biology and Biochemistry* **26:** 1087-1091.

Saleh-Lakha S, Miller M, Campbell RG, Schneider K, Elahimanesh P, Hart MM *et al* (2005). Microbial gene expression in soil: methods, applications and challenges. *Journal of Microbiological Methods* **63:** 1-19.

Skujins J, Tann CC, Borjesson I (1987). Dinitrogen fixation in a montane forest sere determined by 15N2 assimilation and in situ acetylene-reduction methods. *Soil Biol Biochem* **19:** 465-471.

Spiff ED (1973). Non-symbiotic nitrogen fixation in latosols derived from sedimentary and basement complex rocks. *J West Afr Sci Assoc* **18:** 177-186.

Stewart WPD, Fitzgerald GP, Harris RH (1967). In situ studies on nitrogen fixation using acetylene-reduction technique. *Proc Natl Acad Sci USA* **58:** 2071-2078.

Tal S, Okon Y (1985). Production of the reserve material poly-β-hydroxybutyrate and its function in Azospirillum brasilense Cd. *Canadian Journal of Microbiology* **31:** 608-613.

Ueda T, Suga Y, Yahiro N, Matsuguchi T (1995). Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of nifH gene sequences. *J. Bacteriol.* **177:** 1414-1417.

Vergin KL, Urbach E, Stein JL, DeLong EF, Lanoil BD, Giovannoni SJ (1998). Screening of a Fosmid Library of Marine Environmental Genomic DNA Fragments Reveals Four Clones Related to Members of the Order Planctomycetales. *Appl. Environ. Microbiol.* **64:** 3075-3078.

Vitousek PM, Cassman K, Cleveland C, Crews T, Field CB, Grimm NB *et al* (2002). Towards an ecological understanding of biological nitrogen fixation. *Biogeochemistry* **57-58:** 1-45.

Zechmeister-Boltenstern S, Kinzel H (1990). Non-symbiotic nitrogen fixation associated with temperate soils in relation to soil properties and vegetation. *Soil Biol Biochem* **22:** 1075-1084.

Zehr JP, Jenkins BD, Short SM, Steward GF (2003). Nitrogenase gene diversity and microbial community structure: a cross-system comparison. *Environmental Microbiology* **5:** 539-554.

Zehr JP, Mellon MT, Zani S (1998). New Nitrogen-Fixing Microorganisms Detected in Oligotrophic Oceans by Amplification of Nitrogenase (nifH) Genes. *Appl. Environ. Microbiol.* **64:** 3444-3450.

CHAPTER 2

EVIDENCE FOR THE FUNCTIONAL SIGNIFICANCE OF DIAZOTROPH COMMUNITY STRUCTURE IN SOIL

Shi-Fang Hsu and Daniel H. Buckley

The ISME Journal (2009) 3, 124–136; doi:10.1038/ismej.2008.82

Abstract

Microbial ecologists continue to seek a greater understanding of the factors that govern the ecological significance of microbial community structure. Changes in community structure have been shown to have functional significance for processes that are mediated by a narrow spectrum of organisms, such as nitrification and denitrification, but in some cases, functional redundancy in the community seems to buffer microbial ecosystem processes. The functional significance of microbial community structure is frequently obscured by environmental variation and is hard to detect in short-term experiments. We examine the functional significance of free-living diazotrophs in a replicated long-term tillage experiment in which extraneous variation is minimized and N-fixation rates can be related to soil characteristics and diazotroph community structure. Soil characteristics were found to be primarily impacted by tillage management, whereas N-fixation rates and diazotroph community structure were impacted by both biomass management practices and interactions between tillage and biomass management. The data suggest that the variation in diazotroph community structure has a greater impact on N-fixation rates than do soil characteristics at the site. N-fixation rates displayed a saturating response to increases in diazotroph community diversity. These results show that the changes in the community structure of free-living diazotrophs in soils can have ecological significance and suggest that this response is related to a change in community diversity.

Keywords: diversity; ecological; microbial; community; *nifH*; soil.

Introduction

Biological N fixation is the major natural process through which atmospheric N_2 is converted into forms that can be used by plants and animals, contributing 100–290 Tg N per year to the biosphere (Cleveland et al., 1999). Although the majority of N fixation in terrestrial ecosystems is carried out by symbiotic bacteria in association with plants, free-living diazotrophs in soils have been shown to be important contributors to the N budgets of a number of ecosystems (Cleveland et al., 1999). Progress in understanding the ecological significance of free-living diazotrophs has been limited, however, by the fact that many of these organisms are recalcitrant to laboratory cultivation. The *nifH* gene, which encodes a subunit of the nitrogenase enzyme, provides a useful marker that can be used to study the distribution and diversity of diazotrophs without the need for cultivation. Surveys of *nifH* diversity in soil commonly reveal sequence types that correspond to the diverse unidentified diazotrophs (Ueda et al., 1995; Widmer et al., 1999; Piceno and Lovell, 2000; Shaffer et al., 2000; Poly et al., 2001). Evidence indicates that these noncultivated diazotrophs, rather than their cultivated cousins, are the dominant N-fixing organisms in many soil systems (Poly et al., 2001; Hamelin et al., 2002; Tan et al., 2003; Buckley et al., 2007).

The ecological significance of free-living diazotrophs in terrestrial ecosystems can be difficult to constrain as estimates for N fixation by these organisms can vary widely, ranging from 0 to 60 kg Ha^{-1} per year (Cleveland et al., 1999). Several environmental factors have been suggested to influence N fixation in soils including soil moisture, oxygen, pH, C quantity and quality, N availability and the availability of trace elements, such as Mo, Fe and V, Soil moisture, oxygen and pH have fairly straightforward effects on N-fixation rates. Increases in soil moisture (Brouzes et al., 1969; Sindhu et al., 1989) and reductions of oxygen tension (Brouzes et al., 1969;

O'Toole and Knowles, 1973; Kondo and Yasuda, 2003a) generally increase rates, whereas N fixation is not favored in soils of low pH (Roper and Smith, 1991; Limmer and Drake, 1996; Nelson and Mele, 2006). In contrast, the effects of C and N quantity and quality are less consistent. Increases in the availability of labile C generally stimulate N fixation (O'Toole and Knowles, 1973; Keeling et al., 1998; Burgmann et al., 2005; Kondo and Yasuda, 2003a, 2003b), but in other cases have little or no effect (Brouzes et al., 1969; Roper and Smith, 1991; Keeling et al., 1998). Likewise, N availability can have either stimulatory (Azam et al., 1988; Poly et al., 2001) or inhibitory (Koteva et al., 1992; Tan et al., 2003) effects. It is reasonable to expect that the influences of these environmental parameters on N-fixation rates vary as a function of microbial community structure, but the functional significance of diazotrophic community composition remains poorly characterized.

Microbial community composition can have significant quantitative and qualitative impacts on important soil processes. Although soil processes that are widely distributed within the community, such as soil respiration, seem to occur largely independent of changes in community structure (Schimel, 1995), those that are mediated by a specialized subset of the community, such as denitrification (Cavigelli and Robertson, 2000), nitrification (Carney et al., 2004; Hawkes et al., 2005; Webster et al., 2005), methane consumption (Gulledge et al., 1997) and cellulose degradation (Wohl et al., 2004) can be strongly impacted by the community level changes. Two recent studies have cast doubt on whether changes in diazotroph community structure have functional significance. Deslippe et al. (2005) were unable to find a significant association between *nifH* T-RFLP profiles and acetylene reduction assay (ARA)-based N-fixation rates in Arctic tundra in a short-term fertilization experiment. Likewise, Patra et al. (2006) were unable to find a significant association between overall *nifH* DGGE profiles and ARA-based N-fixation rates in a long-term grazing experiment,

though 60% of the variance in N-fixation rates could be correlated to the changes in a subset of the DGGE bands observed.

Agricultural experiments provide an excellent opportunity to study the functional significance of microbial community structure in soil because of the relative simplicity and managed aspect of agricultural systems compared with natural ecosystems. Long-term agricultural experiments are particularly useful in this regard as microbial community structure can require long periods of time to equilibrate with respect to changes in management practice (Buckley and Schmidt, 2001, 2003). Thus, long-term treatments are required to investigate linkages between the composition and function of microbial communities in soil (Reed and Martiny, 2007). In this study, we examine the long-term (>30 years) effects of tillage and biomass management on N fixation, soil characteristics and diazotroph community structure to determine whether N-fixation rates are primarily determined by soil characteristics or whether the changes in community structure have functional significance. Diversity is evaluated as a function of its three main components - richness, evenness and genetic diversity. These three components of diversity, although frequently related, can vary somewhat independently (Magurran, 1988). Differences in community structure are evaluated as a function of both differences in the diversity and composition (that is, operational taxonomic unit (OTU) membership) of the communities being compared.

Materials and Methods

Description of field site and sampling

The long-term tillage experiment was established in 1973 and is located at the William H Miner Institute in Chazy, Clinton County, New York. The sire consists of a 2 × 2 factorial design, testing the effects of tillage and biomass management, and is

arrayed in a randomized complete block with four replicate plots for each treatment. Maize has been grown continuously in all plots following standard agronomic practices for the region (26 kg Ha^{-1} per year fertilizer N). The treatments represent a gradient of soil disturbance with each of two treatments managed with annual tillage (T1 and T3; moldboard plowed and disked), and each of the two treatments managed as no-till systems (T2 and T4). These treatments are subdivided by biomass retention (T3 and T4) or removal (T1 and T2). In biomass removal plots, plant biomass was harvested for silage and only roots and stubble were retained in the field. In biomass retention plots, grain was harvested and all other biomass was retained in the field. In addition, a control site consisting of a never cultivated (NC; for >35 years) grassy field was also sampled. The NC site was immediately adjacent to the agricultural site, was on the same soil series and was managed by monthly mowing.

Soil samples were taken on 1 November 2005. A total of 20 soil cores (2.5 cm diameter and 5 cm depth) were taken across each replicate, and these cores were homogenized and sieved to 2 mm. Each replicate soil sample was split for the analysis of soil characteristics, N-fixation rates and diazotroph community structure. Soil characteristics were determined following standard methods with organic matter determined by loss on ignition, total C and N determined by mass spectrometry, and P, K, Mg, Ca, Fe, Al, Mn, Zn, Cu and NO_3 were measured following NH_4OAc extraction as described previously (Burt, 2004). Samples used for determining potential N-fixation rates were maintained at ambient temperatures, and were processed on the same day that they were sampled as described below. Samples used for analysis of diazotroph community structure were frozen on liquid nitrogen in the field, stored on dry ice for transport and archived at −80 °C.

Measurement of N-fixation rates

The ARA is the most common method for measuring N fixation and is based on the assumption that 3–4 mol acetylene are reduced to ethylene for every mole of N_2 fixed by nitrogenase enzyme (Stewart et al., 1967; Jensen and Cox, 1983). However, conversion factors in the range of 0.022–22 have been reported for terrestrial habitats in which ARA has been standardized with $15N_2$-based measurements (Spiff, 1973; Nohrstedt, 1983; Skujins et al., 1987; Zechmeister-Boltenstern and Kinzel, 1990; Liengen, 1999). Thus, in this study, potential N-fixation rates were measured by $15N_2$ incorporation into soil in relation to controls. This method has been widely applied (Brouzes et al., 1969; O'Toole and Knowles, 1973; Nohrstedt, 1983; Skujins et al., 1987; Montoya et al., 1996) and has traditionally been recommended to verify N-fixation rates in cases where ARA is used (Stewart et al., 1967). Briefly, 5 g soil was placed into 18 × 150 mm Balch tubes (Bellco Glass, Vineland, NJ, USA), and the headspace was replaced with synthetic air containing 20% O_2 and 80% $15N_2$ (>98 atom % 15N, Isotec, Miamisburg, OH, USA). Controls were processed in parallel and received unlabeled N_2 gas. Tubes were incubated horizontally at room temperature in the dark for 9 days. The atom % 15N of soil samples was determined using a Finnigan MAT Delta Plus mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA) plumbed to a Carlo Erba NC2500 elemental analyzer (CE Instruments, Wigan, UK) through a Conflo II open split interface for elemental and isotopic composition of solid samples (Thermo Electron Corporation). The net potential N-fixation rate was calculated from the difference of total 15N in soils receiving $15N_2$ relative to controls. It should be recognized that potential rates can differ from in situ rates because of bottle effects, but such potential rates are commonly useful for assessing relative differences in the activities of microbial communities.

Construction of nifH clone libraries

DNA was extracted from three subsamples of 0.33 g from each soil sample using the PowerSoil DNA Isolation Kit (MoBio Inc., Carlsbad, CA, USA) as per manufacturer's instructions and these samples were pooled. DNA concentrations were determined through the Pico-Green assay (Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions. DNA from replicate samples was pooled with respect to treatment before the construction of *nifH* clone libraries. PCR of *nifH* genes was conducted with primers *nifH*-b1-112F (Burgmann et al., 2004) and *nifH*623R (Steward et al., 2004) in 50 μl volumes containing 70 ng of template DNA with each primer at a concentration of 0.25 μM, NTPs at a concentration of 200 μM, 2.5 mM $MgCl_2$, 0.05% of BSA (New England Biolab, Ipswich, MA, USA), 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), and 1 × PCR buffer (supplied with Taq enzyme). Each PCR consisted of a 95 °C hold for 10 min followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 45 s at 72 °C, and a final extension for 15 min at 72 °C. Three PCR reactions were performed in parallel to the pooled DNA sample from each treatment, these PCR products were combined and gel purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), and then cloned into pCR2.1-TOPO as per manufacturer's instructions (Invitrogen). DNA sequencing was performed using an Applied Biosystems Automated 3730 DNA Analyzer at Cornell University's Biotechnology Resource Center. The nucleotide sequences of the 349 *nifH* gene clones described in this study have been deposited in GenBank under accession numbers: FJ008168:FJ008514.

The suitability of the *nifH*-b1-112F and *nifH*623R primer sets for characterizing the diazotroph community present in our soils was validated empirically. A range of different primer sets were evaluated as described in Burgmann et al. (2004), and of the primer combinations tested the *nifH*-b1-112F and *nifH*623R

provided the best amplification for our samples (data not shown). To confirm that the *nifH*-b1-112F and *nifH*623R primer sets provided a representative measurement of the diazotroph community for our samples, the *nifH* clone library generated with these primers was compared to a *nifH* clone library generated from the pooling DNA amplified in parallel using five different *nifH* PCR primer sets having a range of specificities as described previously (Burgmann et al., 2004). All of these reactions used the reverse primer *nifH*623R and one of the following forward primers: *nifH*-b1-112F, *nifH*-a1-112F, *nifH*-c1-112F, *nifH*-f1-112F and *nifH*-g1-112F. The DNA template used for these PCR was from the NC soil; each reaction was performed in triplicate. The PCR products were then pooled across all primer sets, and this pooled PCR product was used to create a clone library as described above. A total of 70 *nifH* sequences from this library were determined and deposited in GenBank under accession numbers: FJ008515:FJ008582. The Chao1 estimator (see below) revealed a richness of 39 (95% confidence intervals: 29, 74) OTU in the mixed primer library, and 64 (95% confidence intervals: 44, 122) in the library constructed with primers *nifH*-b1-112F and *nifH*623R. The lack of a significant difference in diversity between these libraries suggests that the *nifH*-b1-112F and *nifH*623R primer sets provide a reasonable estimate of diazotroph community structure in soil from our site.

Phylogenetic analysis of nifH sequences

Phylogenetic analysis was performed using ARB (Strunk and Ludwig, 1997) and Phylip 3.64 (Felsenstein, 2005). Sequences were imported and aligned against an *nifH* database constructed from sequences available in Genbank that were aligned against the Pfam Fer4_*NifH* amino-acid seed alignment (Finn et al., 2006). Regions of ambiguous alignment were identified and excluded from subsequent phylogenetic

analyses. Phylogenetic trees were generated by performing protein parsimony (Swofford, 1991), and maximum-likelihood analyses (Olsen et al., 1994).

Analyses of diazotroph community composition

Diversity analyses were performed on aligned DNA sequences of 487 nucleotides in length. OTU classification was performed using DOTUR as described previously (Schloss and Handelsman, 2005). Protein-encoding genes from the strains of a given species generally have an average nucleotide identity, whicht is >93%–95% (Konstantinidis and Tiedje, 2005), and thus a conservative OTU cutoff of 93% similarity was used for diversity calculations. Community structure was evaluated as a function of changes in community diversity and composition as discussed previously (Schloss and Handelsman, 2008). Within community, diversity was evaluated both as richness, as determined by the Chao I estimator (Hughes et al., 2001), and evenness, as calculated by the evenness component of the Shannon index (Pielou's JelouMagurran, 1988). Changes in OTU composition between communities were evaluated with Sorenson's index of similarity (Magurran, 1988), and with UniFrac analysis performed with unweighted data (which ignore the relative abundance of OTUs in the library). In addition, *nifH* clone libraries were compared using daLibshuff (Schloss et al., 2004) and UniFrac analyses (Lozupone and Knight, 2005), which assess overall differences in community structure and are sensitive to the changes in richness, evenness, genetic diversity and the composition of communities as described previously (Schloss and Handelsman, 2008). Guide trees for UniFrac were generated through maximum-likelihood analysis as described above. Distance matrices generated with Unifrac were used to cluster communities using UPGMA and jackknife analysis was used to evaluate to the confidence of tree nodes. UniFrac was also used to perform principal coordinates analysis.

Statistical analyses

Statistical tests were performed using StatView v5.0.1 (SAS Institute Inc., Cary, NC, USA). Analysis of variance models included one-way analysis of variance to evaluate the differences between control (NC) and agricultural sites, and 2 × 2 factorial analysis of variance to evaluate the main effects of tillage and biomass retention among the agricultural sites. In addition, stepwise regression was used to evaluate linear relationships between soil variables and N-fixation rates. Stepwise regression was performed using forward and backward procedures and collinear variables were removed, so that both models produced identical results.

Results

Impacts of tillage and biomass treatments on soil characteristics

Tillage and biomass management were both found to have significant impacts on soil characteristics (Tables 2-1 and 2-2). The effect of three decades of continuous tillage was clearly evident at the site as 12 of the 19 soil characteristics (do not include N fixation) examined varied significantly with respect to tillage and one variable, extractable soil K, demonstrated an interaction between the effects of tillage and biomass management that was significant (Table 2-2). In contrast, the impact of biomass retention was more modest, and the only effects observed were increases in soil P and K content in fields where biomass was retained (Tables 2-1 and 2-2). Ignoring management type, the agricultural treatments differed from NC control sites in all soil characteristics measured except extractable K, Mg and Mn (Table 2-1). Soil moisture ($F_{1,18}$=9.23, P=0.008), organic matter ($F_{1,18}$=17.44, P=0.0006), DNA ($F_{1,18}$=10.263, P=0.0049), total C ($F_{1,18}$=14.553, P=0.013), total N ($F_{1,18}$=21.6, P=0.0002), nitrate ($F_{1,18}$=23.23, P=0.0002), Fe ($F_{1,18}$=18.13, P=0.0005), Al

Table 2-1. Soil and site characteristics of the long term tillage experiment in Chazy, NY as meaured November 2005.

Treatment	*Tillage*	*Biomass Retention*	*N-fixed* $\mu g\ kg^{-1}\ d^{-1}$	*pH*	*Moisture* %	*O. M.* $g\ Kg^{-1}$	*DNA* $\mu g\ g^{-1}$	*Total C* $g\ Kg^{-1}$
T1	Yes	No	382 ± 22	8.0 ± 0.1	20.4 ± 1.4	34.4 ± 3.6	6.4 ± 1.0	16.7 ± 1.8
T2	No	No	392 ± 55	7.6 ± 0.5	22.9 ± 1.5	50.0 ± 9.9	11.0 ± 1.9	23.3 ± 4.9
T3	Yes	Yes	351 ± 41	8.0 ± 0.03	21.0 ± 4.8	42.1 ± 9.3	9.0 ± 1.8	16.9 ± 2.1
T4	No	Yes	190 ± 11	7.7 ± 0.2	25.9 ± 3.6	57.8 ± 8.2	10.0 ± 0.5	27.6 ± 4.4
NC	No	Yes	321 ± 49	6.5 ± 0.3	26.4 ± 2.0	71.0 ± 4.2	15.4 ± 1.8	32.4 ± 2.4

Treatment	*Tillage*	*Biomass Retention*	*Total N* $g\ Kg^{-1}$	NO_3 mg/Kg^{-1}	*P* mg/Kg^{-1}	*K* mg/Kg^{-1}	*Mg* mg/Kg^{-1}	*Fe* mg/Kg^{-}
T1	Yes	No	1.3 ± 0.1	0.6 ± 0.1	8.9 ± 1.0	42.0 ± 6.6	161.8 ± 29.1	1.1 ± 0.7
T2	No	No	1.9 ± 0.4	9.2 ± 6.4	10.5 ± 1.2	38.3 ± 5.5	179.1 ± 42.1	0.6 ± 0.3
T3	Yes	Yes	1.3 ± 0.1	0.6 ± 0.1	12.6 ± 3.8	54.1 ± 13.3	165.2 ± 16.2	0.7 ± 0.8
T4	No	Yes	2.1 ± 0.2	2.7 ± 5.1	13.2 ± 3.3	72.4 ± 8.0	238.9 ± 31.6	0.8 ± 0.4
NC	No	Yes	2.7 ± 0.2	17.0 ± 6.8	1.3 ± 0.7	64.5 ± 9.5	222.9 ± 24.1	3.0 ± 1.9

Treatment	*Tillage*	*Biomass Retention*	*Ca* g/Kg^{-1}	*Al* mg/Kg^{-1}	*Mn* mg/Kg^{-1}	*Zn* mg/Kg^{-1}	*Cu* mg/Kg^{-1}
T1	Yes	No	4.46 ± 1.1	6.2 ± 2.3	14.4 ± 5.2	0.9 ± 0.1	3.9 ± 2.2
T2	No	No	3.60 ± 1.1	4.9 ± 1.3	10.3 ± 1.0	0.9 ± 0.3	2.9 ± 0.3
T3	Yes	Yes	3.88 ± 0.3	5.7 ± 0.6	14.8 ± 2.2	1.1 ± 0.2	3.2 ± 0.7
T4	No	Yes	3.39 ± 0.8	3.6 ± 0.3	10.6 ± 1.3	1.1 ± 0.3	3.0 ± 0.7
NC	No	Yes	2.34 ± 0.2	13.0 ± 7.9	15.2 ± 1.5	1.6 ± 0.4	1.4 ± 0.9

($F_{1,18}$=16.02, P=0.0008) and Zn ($F_{1,18}$=19.49, P=0.0003) were all higher in NC than in agricultural sites (Table 2-1). In contrast, pH ($F_{1,18}$=61.299, P<0.0001), P ($F_{1,18}$=44.39, P<0.0001), Ca ($F_{1,18}$=10.94, P=0.004) and Cu ($F_{1,18}$=9.28, P=0.007) were lower in NC than in agricultural sites (Table 2-1).

Impacts of treatment and soil characteristics on N-fixation rates

Analysis of N-fixation rates in soil revealed that there was an interaction between the effects of tillage and biomass management and the result was significant (Table 2-2). Post hoc tests revealed that this effect was largely driven by the rate of N fixation in the no-till sites with biomass retention (T4), which was significantly lower than that observed in all other sites (Fisher's PLSD, P<0.05). Despite this interaction

effect, the main effect of biomass retention was still observed to cause a reduction in soil N fixation and the result was significant (Table 2-2). Relationships between soil

Table 2-2. Results from ANOVA examining effects of tillage and biomass on soil characteristics presented in Table 1 (and excluding the NC sites).

	N-fixed	*pH*	*Moist.*	*O. M.*	*DNA*	*C*	*N*	*NO_3*	*C/N*	*C/P*
TILLAGE										
M. S.	2.3 x 10^{-8}	0.559	0.028	988	91.9	297	1.96	114.9	0.22	9928
F-Value	2.38	9.6	5.7	13.3	22.4	21.8	32.7	6.95	0.301	5.367
P-Value	0.143	0.008	0.031	0.003	<0.001	<0.001	<0.001	0.022	0.587	0.036
BIOMASS										
M. S.	5.4 x 10^{-8}	0.008	0.004	242	31.8	20.93	0.09	42.8	0.035	287509
F-Value	7.23	0.078	0.577	1.901	3.81	0.629	0.465	2.59	0.049	0.704
P-Value	0.018	0.784	0.46	0.19	0.071	0.441	0.506	0.134	0.828	0.415
TILLAGE x BIOMASS										
M. S.	3.0 x 10^{-8}	0.02	0.003	0.006	0.271	16.61	0.04	41.9	0.615	194206
F-Value	6.8	0.31	0.526	9.59E-05	0.132	1.303	0.676	2.53	0.792	0.581
P-Value	0.030	0.588	0.481	0.992	7.723	0.276	0.427	0.137	0.391	0.646
	N/P	*P*	*K*	*Mg*	*Ca*	*Fe*	*Al*	*Mn*	*Zn*	*Cu*
TILLAGE										
M. S.	1508389	4.55	214	8271	1818	0.108	12.21	68.0	0.001	1.24
F-Value	4.7	0.518	2.72	8.52	2.42	0.312	6.78	7.83	0.009	0.87
P-Value	0.048	0.439	0.125	0.013	0.146	0.587	0.023	0.016	0.926	0.369
BIOMASS										
M. S.	2217	39.46	2129	3987	636	0.054	3.060	0.519	0.20	0.365
F-Value	0.923	6.27	27.05	4.12	0.847	0.155	1.698	0.060	4.27	0.257
P-Value	0.353	0.025	<0.001	0.065	0.375	0.701	0.220	0.811	0.061	0.621
TILLAGE x BIOMASS										
M. S.	431	0.992	484.2	3181	134	0.422	0.679	0.008	0.000	0.567
F-Value	0.222	0.144	6.15	3.28	0.179	1.217	0.377	0.001	0.008	0.400
P-Value	0.646	0.711	0.029	0.095	0.680	0.292	0.551	0.976	0.932	0.539

characteristics and N-fixation rates were explored by both regression and stepwise multiple regression. Soil K was found to be negatively correlated with N-fixation rates and was the only single variable shown to explain significant variation in N-fixation rates (R_2=0.680, P<0.001). Stepwise regression using all independent soil variables identified P and K as the main variables associated with N-fixation rates (R^2=0.747;

$F_{2,18}$ =23.60, P<0.0001), but of these, only K was found to have a significant standardized regression coefficient (−0.826, P<0.0001). If K was removed from the stepwise regression procedure, then a model containing the variables P, Ca and Mg was still found to explain significant variation in N-fixation rates (R^2=0.575; $F_{3,19}$=7.22, P=0.003), and the standardized regression coefficients of these variables (P: −0.599, P=0.004; Mg: −0.544, P=0.004; Ca: 0.453, P=0.023) were significant.

Impacts of treatment and soil characteristics on the diversity of the diazotroph community

The Chao1 estimator was used to assess the richness of diazotrophic communities as revealed through the analysis of *nifH* clone libraries. The accumulation curves indicate that the diazotroph community was well sampled in agricultural sites but may still be under sampled in the NC sites (Figure 2-1). Analysis of *nifH* clone libraries from the five treatments revealed that the no-till plots with biomass retention (T4) had the lowest richness, and the NC sites had the highest richness of all sites examined, and these results were significant (Table 2-3). When pooled by management type within the agricultural sites, differences in the richness of *nifH* with respect to tillage (till: 47±19; no-till: 30±6) or biomass management

Table 2-3. Estimates of *nifH* richness for all sites. OTUs defined by a 93 % similarity cutoff.

	T1	T2	T3	T4	NC
Total sequences	69	66	72	67	75
Observed OTU	16	17	15	8	35
Chao 1 (mean ± SD)	18 ± 2	21± 4	18 ± 3	8 ± 1	64 ± 18
Chao 1 upper 95% C. I.	28.0	39.3	32.6	12.1	121.7
Chao 1 lower 95% C. I.	16.2	17.8	15.5	8.0	44.4

(retention: 26±4; removal: 31±4) were not significant (s.d. of the mean is reported). In contrast, evenness varied considerably across the sites (Figure 2-2) with greater dominance observed in biomass retention plots than in plots where biomass was removed or in the control plots (Figure 2-2).

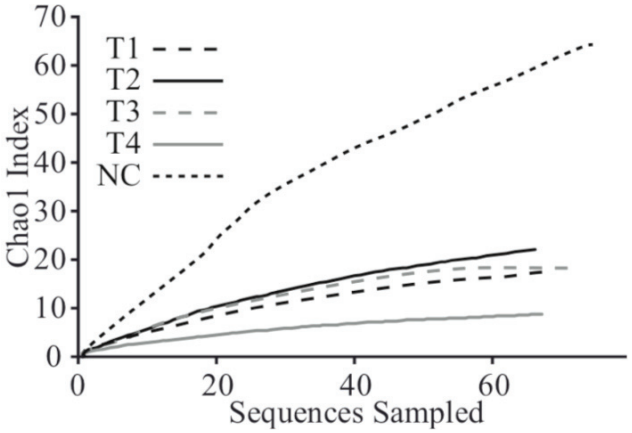

Figure 2-1. Chao I richness estimates for *nifH* clone libraries with OTUs defined by a 93% DNA similarity cutoff. Confidence intervals are provided in Table 2-3. OTUs, operational taxonomic units.

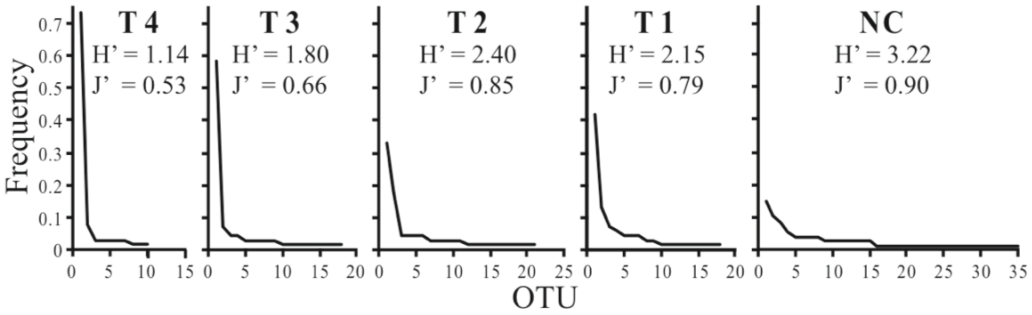

Figure 2-2. Frequency distribution for OTUs observed in *nifH* clone libraries. The label for each panel provides the treatment identifier, the Shannon index of diversity (H') and the evenness component of the Shannon index (Pielou's J') for each library. OTUs, operational taxonomic units.

Impacts of treatment and soil characteristics on the structure of the diazotroph community

Diazotroph community structure differed across the treatments as a function of both biomass and tillage management. Results from ∫-Libshuff analysis of the *nifH* libraries indicated that the community structure did not differ significantly between plots in which biomass was retained (T3 and T4), but there were significant differences in community composition between all other pairwise combinations of treatments ($P<0.05$, after Bonferroni correction). UniFrac analysis of *nifH* clone libraries also supports the conclusion that biomass retention has an impact on the composition of the diazotroph community and the result is significant (Figure 3-3a). When *nifH* sequences were pooled with respect to management type both the effect of biomass retention and tillage were found to have significant impacts on diazotroph community composition (∫-Libshuff, $P<0.05$, after Bonferroni correction). To evaluate the effect that OTU relative abundance has on these analyses, UniFrac analysis was also performed with (Figure 3-3a) and without (Figure 3-3b) respect to OTU abundance in the library. In the unweighted analysis, it was possible to discern the effects of tillage on the composition of the diazotoph community (Figure 3-3b). Likewise, Sorenson's index of similarity, which evaluates the presence or absence of OTUs without respect to abundance, indicated greater similarity between the diazotrophic communities in tilled fields (T1 and T3: 0.92) relative to either no-till fields (T2 and T4: 0.52), plots that shared biomass management (T1 and T2: 0.62; T3 and T4: 0.048), or any comparison of the agricultural and control plots (0.15±0.05, n=4 comparisons). These results are broadly consistent with an interaction of tillage and biomass management on diazotroph community composition in which biomass retention results in an increase in the dominance of certain OTUs (that is, reduced

evenness) in T3 and T4, whereas tillage results in an increase in the number of shared OTUs between T1 and T3.

The impact of treatment effects on the abundance of particular OTUs in *nifH* clone libraries was investigated. The abundance of a set of four OTUs that comprise a monophyletic grouping within a cluster of *nifH* genes most commonly associated with Alphaproteobacteria (Figure 2-4, shaded box) was found to be greater in plots with biomass retention (93 of 139 sequences) than in plots where biomass was removed (55

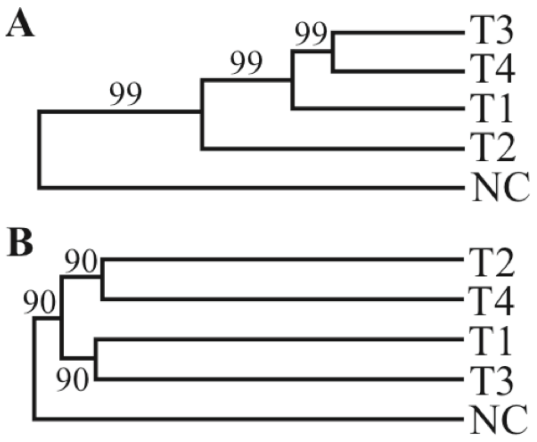

Figure 2-3. Dendograms from UniFrac analyses of *nifH* clone libraries. Numbers indicate the frequency with which nodes were supported by jackknife analysis. Analyses were performed with respect to the abundance of each OTU (weighted data, A), and by ignoring OTU abundance (un-weighted data, B). OTU, operational taxonomic unit.

of 135 sequences) and the result is significant (Fisher's exact test, P=0.0001). The majority of these sequences (82%) fall into a single dominant OTU (Figure 2-4), and when these data are removed, the effect of biomass is still significant (Fisher's exact test, P=0.001). In contrast, the abundance of this group in clone libraries does not vary significantly with respect to tillage (Fisher's exact test, P>0.05). As a result, we can conclude that this single group of *nifH* sequences has become enriched as a result of

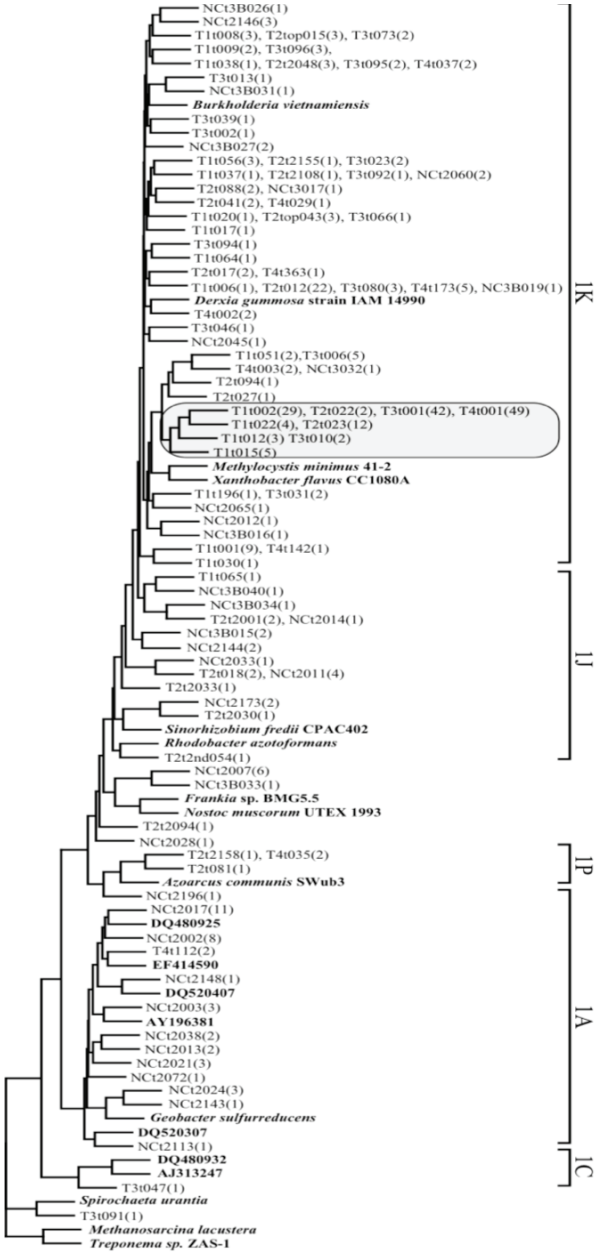

Figure 2-4. Maximum parsimony tree of representative *nifH* sequences described in this study. The tree was constructed from translated data using 109 amino acid positions. Each unique OTU is represented once in the tree. Branch labels contain multiple sequence names to indicate when an OTU occurs in more than one site and a representative sequence from each site is provided. The number in parenthesis following sequence names indicates the number of sequences observed for each OTU in each site. The names of reference sequences not determined in this study are in bold. The shaded box encompasses the dominant group of OTUs as discussed in the text. OTU, operational taxonomic unit.

biomass retention and is largely responsible for the differences observed in the evenness of the diazotrophic community

N-fixation and diazotroph community structure

N fixation rates were observed to vary as a function of the diversity and composition of the diazotrophic community. N-fixation rates displayed a unimodal distribution with respect to both evenness (Figure 5a) and, to a lesser degree, the richness (Figure 5b) of the diazotophic community, and the results were significant. The effect of the lowest diversity sample (T4) on this analysis was examined, and when these datare removed, the relationship between N fixation and evenness remains significant (R^2=0.999, P<0.001), whereas the relationship to richness is no longer significant. A similar relationship between N-fixation rates and diazotrophic community composition was detected as a result of principal component analysis of community similarity conducted using UniFrac analysis (Figure 2-5c). Change in a principal component representing 30% of variation in community membership was found to correlate with differences in N-fixation rate (Figure 2-5c). This analysis was conducted with unweighted data, and is therefore not influenced by the changes in evenness or abundance caused by the dominant OTU found on the agricultural site (as described above).

Discussion

Treatment effects on diazotroph diversity

The experimental site had been established for more than 30 years as a randomized complete block to control for variation in the landscape. The treatments are relatively small in scale with the whole site occupying less than 1 Ha and all plots

having the same soil type and being planted to the same crop for the entire history of the experiment. Thus, extraneous variation is minimized in this experiment relative to many studies that focus on the functional significance of soil communities. In addition, the diversity of the diazotrophic community in soil is typically far lower than that of the overall community, with estimates of *nifH* richness generally between 11–99 OTU 0.03 g^{-1} soil (Bothe et al., 2002; Izquierdo and Nusslein, 2006; Roesch et al., 2008). Regardless, the response of the diazotrophic community to the effects of tillage and biomass management that we observed was complex and differed with respect to the richness, evenness and the composition of the community.

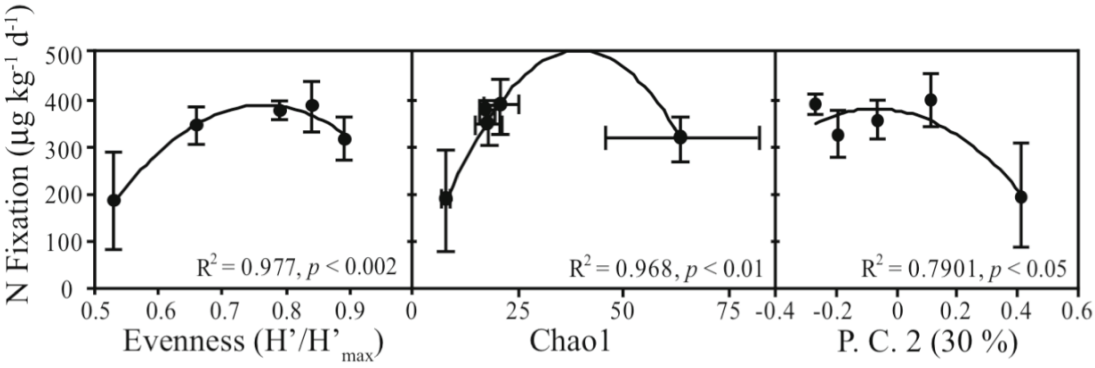

Figure 2-5. Relationship between N-fixation rate and diazotroph diversity as estimated from *nifH* clone libraries and defined by evenness, richness, and community composition. Community evenness (A) is defined by the evenness component of the Shannon index (Pielou's J' = H'/H'_{max}); Community richness (B) is defined by the Chao1 estimator; and community composition (C) was assessed by Unifrac principle coordinate analysis of un-weighted data from *nifH* libraries (and is thus a measure of composition unaffected by differences in evenness). The principle coordinate depicted describes 30% of the variation in *nifH* library composition. Regression was performed with a 2nd order polynomial function consistent with the observation of a unimodal response. Error bars indicate standard deviation of the mean.

Biomass retention caused a dramatic change in the evenness of the diazotroph community (Figure 2-2), and this result was likely responsible for the results obtained by both ∫-Libshuff and weighted UniFrac analyses (Figure 2-3a), which indicated a significant effect of biomass management on diazotroph community structure. This change in evenness was primarily driven by the enrichment of a single group of dominant *nifH* sequences (Figures 2-4) in response to biomass retention. However, ∫-Libshuff indicated that the tillage also had a significant impact on community structure. The effect of tillage became more apparent, when the effects of changes in evenness were removed by performing analyses that ignore OTU abundance (Figure 2-3b). Taken together, these results indicate an interaction between the effects of tillage and biomass retention on diazotroph community structure. The interaction seems to result from the impact of biomass management on evenness (caused by the enrichment of a dominant OTU in response to biomass retention) and the impact of tillage on community composition (caused by the homogenization of community composition in response to tillage of the soil). The richness results also support an interaction effect as data pooled with respect to either tillage or biomass did not reveal significant differences with respect to richness, whereas richness was significantly reduced in no-till biomass retention (T4) plots relative to either tilled biomass retention (T3) plots or no-tilled biomass removal (T2) plots (Table 2-3).

A range of treatment effects can impact the structure of the diazotrophic community in soils including changes in plant community composition (Tan et al., 2003; Patra et al., 2006), burning (Yeager et al., 2005), pH (Nelson and Mele, 2006), soil N (Bothe et al., 2002; Mergel et al., 2001; Tan et al., 2003) and biomass retention (Nelson and Mele, 2006; Wakelin et al., 2007). In this study, plant community composition was held constant across agricultural sites, but differed considerably between those and the control (NC) sites. The effects of pH on diazotroph community

structure have largely been documented to be important at pH values below 5.3 (Limmer and Drake, 1996; Nelson and Mele, 2006), but have not been observed to have effects in the pH range of 6.5–8.0 that characterized the soil from our site. The effects of soil N on diazotroph community structure tend to be somewhat complex. Maximal *nifH* abundance has been observed to coincide with peak values for nitrate and total N (Bothe et al., 2002; Mergel et al., 2001), but fertilization does not necessarily result in an increase in *nifH* abundance (Wakelin et al., 2007). In addition, the changes in diazotroph community structure show no relationship to N availability in upland soils (Shaffer et al., 2000; Poly et al., 2001) or salt marsh soils (Piceno and Lovell, 2000), but do in alpine soils (Zhang et al., 2006) and in paddy soils (Tan et al., 2003).

Impacts of treatment and soil characteristics on N-fixation rates

Given the inconsistent response of free-living diazotrophs to N availability in soil as described above, it is perhaps not surprising that we did not find a relationship between N-fixation rates and soil N or nitrate. In contrast, the presence of high C/N crop biomass clearly has the potential to stimulate N fixation in soils, and biomass retention can impact N fixation through the effects on both soil moisture and temperature (Roper, 1983). Biomass retention or addition can increase N-fixation rates in cereal crops, including wheat, rice and maize (Roper, 1983; Roper et al., 1989, 1994; Gupta et al., 2006), increase *nifH* abundance in soil (Wakelin et al., 2007) and change diazotroph community structure (Nelson and Mele, 2006). In addition, the effect of biomass retention can interact with the effect of tillage, because crop residues have different effects when left on the soil surface than they do when incorporated into the soil by tilling (Roper et al., 1989). The estimates of N fixation in agricultural fields where biomass is retained, range from 0.03–1.6 kg N Ha^{-1} d^{-1}, with an estimate that

optimal conditions can provide a rate of 0.75 kg N Ha^{-1} d^{-1} (Gupta et al., 2006). The average N-fixation rate observed for the agricultural plots at our site was 0.33±0.13 kg N Ha^{-1} d^{-1}.

We observed that the biomass retention had a significant impact on N fixation and that there was an interaction between the effects of biomass and tillage (Table 2-2). As maize residues have a high C/N ratio (30:1–50:1) the a priori expectation would be a positive correlation between biomass inputs and N-fixation rates; however, the reverse was observed (Table 2-1). This unexpected result suggests that the addition of homogeneous residues has different impacts on soil processes (and possibly on soil communities) over long periods of time than it does in short-term experiments (such as those described above). Although total soil C, N, NO_3, C/N, pH, moisture and organic matter content might all be expected to influence N-fixation rates, these variables were not impacted significantly by biomass management (Table 2-2), and did not explain significant variation in N-fixation rates as assessed either by regression or stepwise regression. Biomass retention did cause a significant increase in extractable cP and K (Table 2-2), and variation in these parameters and Mg were found by multiple regression to be negatively correlated with N-fixation rates. As P, K, Ca and Mg are in order, the four most abundant trace elements in plant biomass following C and N, it seems likely that the correlations observed between these variables and N-fixation rates are caused indirectly by their correlation with plant biomass inputs (the different response of extractable Ca is likely explained by the interaction between pH and Ca mineralogy). As biomass retention was observed to decrease N fixation, we would expect soil variables that track biomass addition, such as P and K, to correlate with N fixation. Changes in these soil variables, however, are unlikely to be driving changes in N fixation, as increases in K, P and Mg were correlated with decreases in N fixation. Limitation of P and K can inhibit N-fixation rates (Alahari and Apte, 1998;

Moisander et al., 2003) indirectly by limiting growth, but there is no mechanism known by which the modest increases in K, P and Mg could inhibit N fixation. Thus, changes in soil characteristics caused by biomass retention cannot mechanistically explain the reductions in N-fixation rate that we observed.

Evidence that diazotroph diversity impacts N-fixation rate

Changes in diazotroph community structure mirrored those of N-fixation rates in that they were both primarily impacted by biomass management and also by the interaction of tillage and biomass. Evidence was found that the changes in community structure as assessed by the evenness (Figure 2-5a), and to a lesser extent the richness (Figure 2-5b) and composition (Figure 2-5c) of the diazotroph community, were correlated with the changes in N-fixation rates. These results show that changes in diazotroph community structure at the site have functional significance and suggest that N-fixation rates vary as a function of the diversity of the diazotroph community. The ecological significance of community diversity has been investigated widely in plant communities and to a lesser degree in microbial systems. The general focus of many of these studies is the influence of diversity on productivity. The most common observation at local and regional scales is a unimodal or hump-shaped relationship between plant community diversity and productivity (for reviews see (Symstad et al., 2003; Hooper et al., 2005)). This observation fits with the general expectation of a saturating response of ecosystem properties to increasing diversity (Hooper et al., 2005). Thus, the observation of a unimodal relationship between N fixation and diazotroph diversity (Figure 2-5a and b) fits existing theory pertaining to the potential significance of diversity on ecosystem properties. It can, however, be difficult to disentangle the ecological significance of changes in the diversity and the composition of communities. The effect of biomass retention on the evenness and richness of the

diazotroph community is clearly related to the changes in the abundance of the dominant OTU at the site. The distribution and activity of highly dominant species have previously been shown to exert large impacts on ecosystem function in plant communities (Symstad et al., 2003). These impacts can be caused either directly by the activity of the dominant species, or indirectly through the impacts of dominant species on the diversity or activity of the rest of the community (Symstad et al., 2003). It should be noted, however, that a relationship between community composition and N-fixation rate was also observed when OTU abundance (and hence evenness) was ignored (Figure 2-5c), demonstrating that the relationship between community structure and N-fixation rates is not solely a function of the distribution of any one OTU at the site.

Problems associated with inferring the ecological significance of community composition from environmental treatments have been reviewed previously (Reed and Martiny, 2007). Two common problems encountered are that experimental treatments are not maintained for sufficient time to allow for community composition to reach equilibrium, and that the effects of community composition on a given ecological process cannot be resolved from those of the treatment itself. The current experiment has been maintained for more than 30 years, and so the former concern does not seem immediately relevant, but the latter requires some consideration. Biomass and tillage treatments would primarily impact N-fixation rates through their impacts on soil characteristics; however, variation in the soil characteristics observed at the site did not satisfactorily explain N-fixation rates. The quality of biomass inputs can impact N-fixation rates (Vitousek and Hobbie, 2000), but all C inputs derived from this experiment were from maize, and only varied in quantity between treatments. One potentially confounding variable in this study is the effect of soil physical structure on the community. Aggregate stability has been observed to vary as a function of both

tillage and biomass at our site (Harold van Es, personal communication), and soil aggregate size can have a profound impact on both N-fixation rates and on diazotrophic community composition (Poly et al., 2001; Chotte et al., 2002). However, changes in community structure and N-fixation rates are likely to be intimately related within soil aggregates and difficult to disentangle. As a result, it seems clear that the changes in community structure have functional significance at the site, but it is not clear whether this response is mechanistically explained by a change in community diversity itself or from treatment-induced changes in soil structure and their resulting impacts on community structure and function.

Future perspectives

A final consideration is the degree to which N-fixation rates measured at a single time are ecologically significant or the degree to which N-fixation rates or diversity may vary temporally at the site. Research is currently ongoing to address this issue as N-fixation rates, soil characteristics and diazotroph community structure in the sites have been monitored over a period of 2 years. Preliminary evidence suggests that when averaged over time, N fixation continues to be depressed in plots where biomass is retained relative to where it is removed (data not shown). It is expected that measurement over time of changes in the abundance and composition of the diazotroph community in relation to N-fixation rates will make it possible to better constrain the functional significance of community composition. We have shown that the changes in rates of N fixation are related to the changes in diazotroph community structure and cannot be satisfactorily explained solely by the soil variables of the site. These results show that the changes in the community structure of free-living diazotrophs in soils can have ecological significance and suggest that this response is related to a change in community diversity.

Acknowledgements

This research was supported by the National Science Foundation under award number MCB-0447586. This research was also supported in part by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant number 2005-35107-15266.

REFERENCE

Alahari A, Apte SK. (1998). Pleiotropic effects of potassium deficiency in a heterocystous, nitrogen-fixing cyanobacterium, Anabaena torulosa. *Microbiolog-SGM* **144**: 1557–1563.

Azam F, Mahmood T, Malik KA. (1988). Immobilizationremineralization of NO_3-N and total N balance during the decomposition of glucose, sucrose and cellulose in soil incubated at different moisture regimes. *Plant Soil* **107**: 159–163.

Bothe H, Mergel A, Rosch C. (2002). Diversity of dinitrogen fixing and denitrifying bacteria in soils assessed by molecular biological methods. In: Finan TM, O'Brian MR, Layzell DB, Vessey JK, Newton W (ed). *Nitrogen Fixation: Global Perspectives*. CABI Publishing: New York. pp 305–308.

Brouzes R, Lasik J, Knowles R. (1969). Effect of Organic Amendment, Water Content, and Oxygen on Incorporation of $15N_2$ by Some Agricultural and Forest Soils. *Can J Microbiol* **15**: 899–905.

Buckley DH, Huangyutitham V, Hsu S-F, Nelson TA. (2007). Stable Isotope Probing with $15N_2$ reveals novel non-cultivated diazotrophs in soil. *Appl Environ Microbiol* **73**: 3196–3204.

Buckley DH, Schmidt TM. (2001). The structure of microbial communities in soil and the lasting impacts of cultivation. *Microb Ecol* **42**: 11–21.

Buckley DH, Schmidt TM. (2003). Diversity and dynamics of microbial communities in soils from agroecosystems. *Environ Microbiol* **5**: 441–452.

Burgmann H, Meier S, Bunge M, Widmer F, Zeyer J. (2005). Effects of model root exudates on structure and activity of a soil diazotroph community. *Environ Microbiol* **7**: 1711–1724.

Burgmann H, Widmer F, Von Sigler W, Zeyer J. (2004). New molecular screening tools for analysis of freeliving diazotrophs in soil. *Appl Environ Microbiol* **70**: 240–247.

Burt R. (2004). Soil Survey Laboratory Methods Manual Version 4 ed. vol. Soil Survey Investigations Report No. 42. National Resources Conservation Service.
Carney KM, Matson PA, Bohannan BJM. (2004). Diversity and composition of tropical soil nitrifiers across a plant diversity gradient and among land-use types. *Ecology Lett* 7: 684–694.

Cavigelli MA, Robertson GP. (2000). The functional significance of denitrifier community composition in a terrestrial ecosystem. *Ecology* **81**: 1402–1414.

Chotte JL, Schwartzmann A, Bally R, Monrozier LJ. (2002). Changes in bacterial communities and Azospirillum diversity in soil fractions of a tropical soil under 3 or 19 years of natural fallow. *Soil Biol Biochem* **34**: 1083–1092.

Cleveland CC, Townsend AR, Schimel DS, Fisher H, Howarth RW, Hedin LO et al. (1999). Global patterns of terrestrial biological nitrogen (N_2) fixation in natural ecosystems. *Global Biogeochem Cy* **13**: 623–645.

Deslippe JR, Egger KN, Henry GHR. (2005). Impacts of warming and fertilization on nitrogen-fixing microbial communities in the Canadian High Arctic. *FEMS Microbiol Ecol* **53**: 41–50.

Felsenstein J. (2005). PHYLIP (Phylogeny Inference Package), v3.64. University of Washington, Department of Genome Sciences: Seattle.

Finn RD, Mistry J, Schuster-Böckler B, Griffiths-Jones S, Hollich V, Lassmann T et al. (2006). Pfam: clans, web tools and services. Nucleic Acids Res **34**: D247–D251.

Gulledge J, Doyle AP, Schimel JP. (1997). Different NH_4^+ inhibition patterns of soil CH4 consumption: a result of distinct CH4 oxidizer populations across sites? *Soil Biol Biochem* **29**: 13–21.

Gupta V, Roper MM, Roget DK. (2006). Potential for nonsymbiotic N_2-fixation in different agroecological zones of southern Australia. Aust J Soil Res **44**: 343–354.

Hamelin J, Fromin N, Tarnawski S, Teyssier-Cuvelle S, Aragno M. (2002). *nifH* gene diversity in the bacterial community associated with the rhizosphere of Molinia coerulea, an oligonitrophilic perennial grass. *Environ Microbiol* **4**: 477–481.

Hawkes CV, Wren IF, Herman DJ, Firestone MK. (2005). Plant invasion alters nitrogen cycling by modifying the soil nitrifying community. *Ecology Lett* **8**: 976–985.

Hooper DU, Chapin FS, Ewel JJ, Hector A, Inchausti P, Lavorel S et al. (2005). Effects of biodiversity on ecosystem functioning: A consensus of current knowledge. *Ecol Monogr* **75**: 3–35.

Hughes JB, Hellman JJ, Ricketts TH, Bohannan BJM. (2001). Counting the uncountable: statistical approaches to estimating microbial diversity. *Appl Environ Microbiol* **67**: 4399–4406.

Izquierdo JA, Nusslein K. (2006). Distribution of extensive *nifH* gene diversity across physical soil microenvironments. *Microb Ecol* **51**: 441–452.

Jensen BB, Cox RP. (1983). Direct measurements of steady-state kinetics of cyanobacterial N2 uptake by membrane-leak mass spectrometry and comparisons between nitrogen-fixation and acetylene reduction. *Appl Environ Microbiol* **45**: 1331–1337.

Keeling AA, Cook JA, Wilcox A. (1998). Effects of carbohydrate application on diazotroph populations and nitrogen availability in grass swards established in garden waste compost. *Bioresour Technol* **66**: 89–97.

Kondo M, Yasuda M. (2003a). Effects of temperature, water regime, light, and soil properties on N-15_2 fixation associated with decomposition of organic matter in paddy soils. *Jpn Agric Res Q* **37**: 113–119.

Kondo M, Yasuda M. (2003b). Seasonal changes in N-2 fixation activity and N enrichment in paddy soils as affected by soil management in the northern area of Japan. *Jpn Agric Res Q* **37**: 105–111.

Konstantinidis KT, Tiedje JM. (2005). Genomic insights that advance the species definition for prokaryotes. *Proc Natl Acad Sci USA* **102**: 2567–2572.

Koteva ZV, Kudeyarov VN, Myakshina TN. (1992). Effect of nitrogen fertilizers and other factors on potential nitrogen-fixation and denitrification activity of Gray Forest soil. *Eurasian Soil Sci* **24**: 1–11.

Liengen T. (1999). Conversion factor between acetylene reduction and nitrogen fixation in free-living cyanobacteria from high arctic habitats. *Can J Microbiol* **45**: 223–229.

Limmer C, Drake HL. (1996). Non-symbiotic N-2-fixation in acidic and pH-neutral forest soils: Aerobic and anaerobic differentials. *Soil Biol Biochem* **28**: 177–183.

Lozupone C, Knight R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* **71**: 8228–8235.

Magurran AE. (1988). Ecological diversity and its measurement. Princeton University Press: Princeton, NJ, pp 81–101.

Mergel A, Kloos K, Bothe H. (2001). Seasonal fluctuations in the population of denitrifying and N_2-fixing bacteria in an acid soil of a Norway spruce forest. *Plant Soil* **230**: 145–160.

Moisander PH, Steppe TF, Hall NS, Kuparinen J, Paerl HW. (2003). Variability in nitrogen and phosphorus limitation for Baltic Sea phytoplankton during nitrogen-fixing cyanobacterial blooms. *Mar Ecol Prog Ser* **262**: 81–95.

Montoya JP, Voss M, Kahler P, Capone DG. (1996). A simple, high-precision, high-sensitivity tracer assay for N-2 fixation. *Appl Environ Microbiol* **62**: 986–993.

Nelson DR, Mele PM. (2006). The impact of crop residue amendments and lime on microbial community structure and nitrogen-fixing bacteria in the wheat rhizosphere. *Aus J Soil Res* **44**: 319–329.

Nohrstedt HO. (1983). Conversion factor between acetylene reduction and nitrogen fixation in soil: effects of water content and nitrogenase activity. *Soil Biol Biochem* **15**: 275–279.

Olsen GJ, Matsuda H, Hagstrom R, Overbeek R. (1994). FasDNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comput Appl Biosci* **10**: 41–48.

O'Toole P, Knowles R. (1973). Efficiency of acetylene reduction (nitrogen fixation) in soil: effect of type and concentration of available carbohydrate. *Soil Biol Biochem* **5**: 789–797.

Patra AK, Abbadie L, Clays-Josserand A, Degrange V, Grayston SJ, Guillaumaud N et al. (2006). Effects of management regime and plant species on the enzyme activity and genetic structure of N-fixing, denitrifying and nitrifying bacterial communities in grassland soils. *Environ Microbiol* **8**: 1005–1016.

Piceno YM, Lovell CR. (2000). Stability in natural bacterial communities: I. Nutrient addition effects on rhizosphere diazotroph assemblage composition. *Microb Ecol* **39**: 32–40.

Poly F, Ranjard L, Nazaret S, Gourbiere F, Monrozier LJ. (2001). Comparison of *nifH* gene pools in soils and soil microenvironments with contrasting properties. *Appl Environ Microbiol* **67**: 2255–2262.

Reed HE, Martiny JBH. (2007). Testing the functional significance of microbial composition in natural communities. *FEMS Microbiol Ecol* **62**: 161–170.

Roesch LFW, Camargo FAO, Bento FM, Triplett EW. (2008). Biodiversity of diazotrophic bacteria within the soil, root and stem of field-grown maize. *Plant Soil* **302**: 91–104.

Roper MM. (1983). Field-Measurements of Nitrogenase Activity in Soils Amended with Wheat Straw. *Aust J Agric Res* **34**: 725–739.

Roper MM, Marschke GW, Smith NA. (1989). Nitrogenase Activity (C_2H_2 Reduction) in Soils Following Wheat Straw Retention - Effects of Straw Management. *Aust J Agric Res* **40**: 241–253.

Roper MM, Smith NA. (1991). Straw decomposition and nitrogenase activity (C_2H_2 Reduction) by free-living microorganisms from soil - effects of pH and clay content. *Soil Biol Biochem* **23**: 275–283.

Roper MM, Turpin JE, Thompson JP. (1994). Nitrogenase activity (C2h2 Reduction) by free-living bacteria in soil in a long-term tillage and stubble management experiment on a vertisol. *Soil Biol Biochem* **26**: 1087–1091.

Schimel J. (1995). Ecosystem consequences of microbial diversity and community structure. In: Chapin FS, Korner C (ed). Artic and Alpine Diversity: Patterns, Causes, and Ecosystem Consequences. Springer: New York, pp 241–254.

Schloss PD, Handelsman J. (2005). Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl Environ Microbiol* **71**: 1501–1506.

Schloss PD, Handelsman J. (2008). A statistical toolbox for metagenomics: assessing functional diversity in microbial communities. *BMC Bioinformatics* **9**: 34.

Schloss PD, Larget BR, Handelsman J. (2004). Integration of microbial ecology and statistics: a test to compare gene libraries. *Appl Environ Microbiol* **70**: 5485–5492.

Shaffer BT, Widmer F, Porteous LA, Seidler RJ. (2000). Temporal and spatial distribution of the nifH gene of N_2 fixing bacteria in forests and clearcuts in western Oregon. *Microb Ecol* **39**: 12–21.

Sindhu SS, Grover V, Narula N, Lakshminarayana K. (1989). Occurrence of multiple antibiotic-resistance in Azotobacter-Chroococcum. *Zentralblatt Fur Mikrobiologie* **144**: 97–101.

Skujins J, Tann CC, Borjesson I. (1987). Dinitrogen fixation in a montane forest sere determined by $15N_2$ assimilation and in situ acetylene-reduction methods. *Soil Biol Biochem* **19**: 465–471.

Spiff ED. (1973). Non-symbiotic nitrogen fixation in latosols derived from sedimentary and basement complex rocks. *J West Afr Sci Assoc* **18**: 177–186.

Steward GF, Jenkins BD, Ward BB, Zehr JP. (2004). Development and testing of a DNA microarray to assess nitrogenase (nifH) gene diversity. *Appl Environ Microbiol* **70**: 1455.

Stewart WPD, Fitzgerald GP, Harris RH. (1967). In situ studies on nitrogen fixation using acetylene-reduction technique. *Proc Natl Acad Sci USA* **58**: 2071–2078.

Strunk O, Ludwig W. (1997). ARB: a software environment for sequence data, v2.5. Department of Microbiology, Technical University of Munich: Munich, Germany.

Swofford DL. (1991). PAUP: phylogenetic analysis using parsimony, v3.1. Illinois Natural History Survey: Champaign.

Symstad AJ, Chapin FS, Wall DH, Gross KL, Huenneke LF, Mittelbach GG et al. (2003). Long-term and large-scale perspectives on the relationship between biodiversity and ecosystem functioning. *Bioscience* **53**: 89–98.

Tan XY, Hurek T, Reinhold-Hurek B. (2003). Effect of N-fertilization, plant genotype and environmental conditions on nifH gene pools in roots of rice. *Environ Microbiol* **5**: 1009–1015.

Ueda T, Suga Y, Yahiro N, Matsuguchi T. (1995). Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* genesequences. *J Bacteriol* **177**: 1414–1417.

Vitousek PM, Hobbie S. (2000). Heterotrophic nitrogen fixation in decomposing litter: Patterns and regulation. *Ecology* **81**: 2366–2376.

Wakelin SA, Colloff MJ, Harvey PR, Marschner P, Gregg AL, Rogers SL. (2007). The effects of stubble retention and nitrogen application on soil microbial community structure and functional gene abundance under irrigated maize. *FEMS Microbiol Ecol* **59**: 661–670.

Webster G, Embley TM, Freitag TE, Smith Z, Prosser JI. (2005). Links between ammonia oxidizer species composition, functional diversity and nitrification kinetics in grassland soils. *Environ Microbiol* **7**: 676–684.

Widmer F, Shaffer BT, Porteous LA, Seidler RJ. (1999). Analysis of nifH gene pool complexity in soil and litter at a Douglas fir forest site in the Oregon Cascade Mountain Range. *Appl Environ Microbiol* **65**: 374–380.

Wohl DL, Arora S, Gladstone JR. (2004). Functional redundancy supports biodiversity and ecosystem function in a closed and constant environment. *Ecology* **85**: 1534–1540.

Yeager CM, Northup DE, Grow CC, Barns SM, Kuske CR. (2005). Changes in nitrogen-fixing and ammonia-oxidizing bacterial communities in soil of a mixed conifer forest after wildfire. *Appl Environ Microbiol* **71**: 2713–2722.

Zechmeister-Boltenstern S, Kinzel H. (1990). Non-symbiotic nitrogen fixation associated with temperate soils in relation to soil properties and vegetation. *Soil Biol Biochem* **22**: 1075–1084.

Zhang YG, Li DQ, Wang HM, Xiao QM, Liu XD. (2006). Molecular diversity of nitrogen-fixing bacteria from the Tibetan Plateau, China. *FEMS Microbiol Lett* **260**: 134–142.

CHAPTER 3

QUANTIFYING THE RELATIVE CONTRIBUTIONS OF DIAZOTROPH COMMUNITY COMPOSITION AND SOIL CHARACTERISTICS ON NITROGEN FIXATION RATES

Introduction

Biological Nitrogen Fixation (BNF) is an essential process in the biosphere through which atmospheric N_2 gas is converted into forms that are available to plants and animals. The organisms which carry out this process are called diazotrophs and nitrogenase is the enzyme which catalyzes nitrogen fixation. BNF is often the only major N input for oligotrophic ecosystems, including the open ocean and arid systems (van Dommelen and Vanderleyden, 2007). In terrestrial systems, diazotrophs are estimated to contribute 100-290 Tg of N annually (Cleveland *et al.*, 1999). Most of our current understanding of BNF in terrestrial systems derives from studies of plant symbiotic diazotrophs and fixed N derived from symbiotic N-fixation has clear importance in agricultural systems (Newton, 2008). While free-living diazotrophs have been examined extensively in marine systems (Howarth *et al.*, 1988; Patriquin and Knowles, 1972) much less is known about the diversity or significance of these organisms in soils. Dinitrogen reductase also known as the iron protein subunit of nitrogenase is encoded by *nifH* (Raymond *et al.*, 2004) and this gene is the most common biomarker used for cultivation independent analysis of diazotrophic communities (Zehr *et al.*, 2003). There are currently more than 12,000 *nifH* DNA sequences in Genbank, the majority of which are derived from uncultivated and unidentified diazotrophs (unpublished data). Further studies of these non-cultivated free-living diazotrophs are needed to understand the factors that impact the diversity and activity of these organisms and their potential contributions to terrestrial systems.

In general, the role of microbial community composition as a quantitative and qualitative determinant of ecological processes remains actively debated. Hypotheses postulate relationships between the diversity of functional groups and/or communities and the rates of environmental processes (Reed and Martiny, 2007; Waide *et al.*, 1999). Studies of particular functional groups can help to provide insight on the potential of microbial diversity to impact ecological processes. As might be expected, the relationship between dizaotrophic community structure and N-fixation rates in soil remain largely uncharacterized. Previous studies of diazotroph community composition on N-fixation rates have focused on either symbiotic systems or on free-living organisms that are easily cultivated in the laboratory (Howard and Rees, 1996; Leigh, 2002; Postgate, 1998). Since non-cultivated diazotrophs dominate in most soils relative to symbiotic and easily cultivatable strains it is important to use cultivation independent techniques to evaluate diazotroph community composition in relation to N-fixation rates.

In order to evaluate the relationship between diazotroph community structure and N-fixation rates in soil we used *nifH* terminal restriction fragment length polymorphisms (TRFLP) to characterize diazotrophic community composition in soil in relation to soil N-fixation measurements. TRFLP is a molecular fingerprinting technique (Liu *et al.*, 1997) which is commonly used for studying relationships between microbial community composition and environmental factors (Blackwood *et al.*, 2003; Meier *et al.*, 2008). The long term agricultural experiment site in Chazy, Clinton County, New York was used for this study. We examined variation in *nifH* TRFLP, soil characteristics, and N-fixation rates with respect to tillage and biomass management practices at the site at 6 times over a period of 2 years. The data were analyzed using a general linear model (GLM) to evaluate the degree to which variation

in community composition and soil characteristics were related to variation in rates of N-fixation in soil.

Materials and Methods

Description of field site and sampling

This study was conducted using samples from research plots maintained at the William H. Miner Institute in Chazy, Clinton County, New York (45º 53.13'-73º 28.40') (Hsu and Buckley, 2009; Moebius-Clune *et al.*, 2008). All of the plots share a soil type, which is Raynham Silt Loam. The research plots have been maintained as a long term tillage experiment since 1973. The site is established as a 2 by 2 factorial design with four replicates in a randomized block and represents a gradient of soil disturbance with two treatments subjected to annual tillage with moldboard plow and disked (T1 and T3); and two no-till treatments (T2 and T4). These treatments are further subdivided by biomass retention with grain removing only (T3 and T4) or biomass removal with all maize residue removal (T1 and T2) after harvest. Maize has been grown continuously on all 4 of these treatments for the entire period of the experiment. In addition, a control site consisting of a never cultivated (for >35 years) grassy field (NC) was also sampled. The NC site was immediately adjacent to the agricultural site, was on the same soil series, and was managed by monthly mowing. For description purpose, annual tillage and no-till refer to tillage treatments; biomass retention and removal refer to biomass treatments in this propose.

Soil samples were taken on six different time points during 2005 to 2006 (lists in Table 3-1). A total of 20 soil cores (2.5 cm diameter and 5 cm depth) were taken across each replicate and these cores were homogenized and sieved to 2 mm. Each

replicate soil sample was split for analysis of soil characteristics, N-fixation rates and diazotroph community structure. Soil characteristics were determined following

Table 3-1. Soil and site characteristics of the long-term tillage experiment in Chazy, NY. Measured in 6 time points.

Treatments	*Times*	*Tillage*	*Biomass retention*	*N-fixation* $mg\ kg^{-1}\ day^{-1}$	*DNA* $mg\ g^{-1}$	*Total C* $g\ kg^{-1}$	*Total N* $g\ kg^{-1}$	*Moisture (%)*
NC	*2005/09/09*	N	N	0.100±0.043	9.74±1.14	29.1±3.7	2.6±0.3	36.8±2.8
	2005/11/01	N	N	0.321±0.049	10.27±0.06	28.1±2.2	2.7±0.1	28.3±2
	2006/05/24	N	N	0.126±0.098	11.05±3.27	32±1.2	2.9±0.1	36.9±1.4
	2006/08/10	N	N	0.376±0.033	7.51±0.23	33.1±1.5	3.1±0.2	24.1±0.7
	2006/09/24	N	N	0.398±0.056	10.27±0	31.3±3.3	2.7±0.2	22.1±1.7
	2006/11/05	N	N	0.276±0.066	10.27±0.06	31.2±1.9	3±0.1	34.7±1.6
T1	*2005/09/09*	Y	N	0.275±0.021	4.9±0.75	14.9±2.1	1.2±0.2	23.6±2
	2005/11/01	Y	N	0.382±0.022	4.65±1.73	14.7±1.7	1.2±0	20.3±1.4
	2006/05/24	Y	N	0.274±0.041	9.08±2.34	16.5±2.6	1.3±0.2	25.4±2.5
	2006/08/10	Y	N	0.253±0.137	3.24±1.01	16.5±2.6	1.4±0.2	17.8±1.5
	2006/09/24	Y	N	0.424±0.109	9.67±1.17	19.4±2.6	1.5±0.2	25.5±0.9
	2006/11/05	Y	N	0.231±0.057	4.65±1.73	16.6±3.1	1.4±0.2	24.4±4.2
T2	*2005/09/09*	N	N	0.310±0.025	7.92±1.38	21.8±3.3	1.8±0.2	27.8±0.5
	2005/11/01	N	N	0.392±0.055	7.68±1.02	18.9±3.1	1.8±0.3	22.9±1.5
	2006/05/24	N	N	0.286±0.018	12.74±2.37	22.4±3.7	2.1±0.3	27.2±2.2
	2006/08/10	N	N	0.386±0.010	5.93±1.84	23.6±3.6	2.1±0.3	21±1.2
	2006/09/24	N	N	0.368±0.037	9±2.54	19.1±3.7	1.7±0.3	28.4±4.7
	2006/11/05	N	N	0.297±0.014	7.68±1.02	23.2±3.7	2±0.4	28.1±2.9
T3	*2005/09/09*	Y	Y	0.302±0.048	6.79±1.26	15.3±4	1.2±0.3	24.3±1.5
	2005/11/01	Y	Y	0.351±0.041	5.32±2.06	14.2±2.9	1.3±0.3	20.9±4.8
	2006/05/24	Y	Y	0.282±0.052	15.15±2.21	16.6±3.3	1.4±0.3	28.7±5
	2006/08/10	Y	Y	0.346±0.009	4.64±1.11	18.1±2.4	1.5±0.1	18.8±1.1
	2006/09/24	Y	Y	0.312±0.059	8.43±2.26	15.3±1.7	1.3±0.1	20.8±5.9
	2006/11/05	Y	Y	0.174±0.078	5.32±2.06	19.4±4.8	1.5±0.2	27.8±2.2
T4	*2005/09/09*	N	Y	0.319±0.036	9.58±0.56	24.6±5.7	2.1±0.4	31.8±1.8
	2005/11/01	N	Y	0.190±0.110	8.68±1.42	21.9±4.6	2±0.3	24±0.7
	2006/05/24	N	Y	0.265±0.075	15.85±7.44	27.6±4.7	2.3±0.4	32.7±5.3
	2006/08/10	N	Y	0.421±0.141	4.96±1.9	28±3	2.3±0.2	22.9±1.2
	2006/09/24	N	Y	0.262±0.104	9.54±1.37	19.7±5.1	1.7±0.4	21.6±1.2
	2006/11/05	N	Y	0.266±0.025	8.68±1.42	30.7±6	2.3±0.3	31.6±3.4

standard methods with organic matter determined by loss on ignition, total C and N determined by mass spectrometry, and P, K, Mg, Ca, Fe, Al, Mn, Zn, Cu and NO_3 measured following NH_4OAc extraction as previously described (Burt, 2004). Samples used for determining potential N-fixation rates were maintained at ambient

temperatures and were processed in the same day they were sampled as described below. Samples used for analysis of diazotroph community structure were frozen on liquid nitrogen in the field, stored on dry ice for transport, and archived at -80°C.

Measurement of N-fixation rates

In this study potential N-fixation rates were measured by $^{15}N_2$ incorporation into soil in relation to controls. This method has been widely applied (Brouzes *et al.*, 1969; Montoya *et al.*, 1996; Nohrstedt, 1983; Skujins *et al.*, 1987) and has traditionally been recommended to verify N-fixation rates in cases where ARA is used. Detail of this method was described in Hsu and Buckley 2009 (Chapter 2). Briefly, 5 g soil of each replicate from each treatment was placed into 18 × 150 mm Balch tubes (Bellco Glass, Vineland, New Jersey), and the headspace was replaced with synthetic air containing 20% O_2 and 80% $^{15}N_2$ (>98 atom % ^{15}N, Sigma-Aldrich/Isotec). Controls were processed in parallel and received unlabeled N_2 gas. Tubes were incubated horizontally at room temperature in the dark for 9 days. The atom % ^{15}N of soil samples was determined using a Finnigan MAT Delta Plus mass spectrometer (Thermo Electron Corporation, Waltham, Massachusetts) plumbed to a Carlo Erba NC2500 elemental analyzer (CE Instruments, Wigan, UK) through a Conflo II open split interface for elemental and isotopic composition of solid samples (Thermo Electron Corporation). The net potential N-fixation rate was calculated from the difference of total ^{15}N in soils receiving $^{15}N_2$ relative to controls. It should be recognized that potential rates can differ from in situ rates due to bottle effects, but such potential rates are commonly useful for assessing relative differences in the activities of microbial communities.

nifH T-RFLP analysis of DNA from soil samples

DNA was extracted from 0.35 g of each soil sample using the PowerSoil™ DNA Isolation Kit (MoBio, Inc, Carlsbad, California) as per manufacturer's instructions. DNA concentrations were determined through the Pico-Green assay (Invitrogen, Carlsbad, California) as per manufacturer's instructions. PCR of *nifH* genes was conducted with primers nifH-b1-112F (Burgmann *et al.*, 2004) which labeled with carboxyfluorescein (FAM) on 5' end and nifH623R (Steward *et al.*, 2004) (primers were synthesed by Invitrogen, Carlsbad, California). Each PCR reaction was performed in 50 µl volumes containing 10 µl of template DNA from soil DNA of each replicate, with each primer at a concentration of 0.25 µM, each dNTP (Promega, Madison, Wisconsin) at a concentration of 200 µM, 2.5 mM $MgCl_2$, 0.05% of BSA (New England BioLabs, Ipswich, MA), 2.5 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, California), and 1x Gold buffer (supplied with AmpliTaq enzyme). Each PCR consisted of a 95°C hold for 10 min followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 45 s at 72°C; and a final extension for 15 min at 72°C. Eight PCR reactions were performed in parallel for each replicate soil sample from each treatment. The expected length of the PCR product was between 470bp to 500bp. These PCR products were combined and gel purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin). After gel purification, clean *nifH* PCR products were quantified by PICO green assay and reconstituted to final concentration of 30 ng/µl. 10 µl of each reconstituted PCR product was digested in a 30 µl reaction using HypCH4IV (A'CGT) (New England BioLabs, Ipswich, MA) in the following reaction mixture: 1× reaction NEBuffer I (came with enzyme), 0.3 µg BSA, 1U HypCH4VI and DNA-free water. Samples were digested for 6 h at 37ºC, and then the enzyme was inactivated by heating for 30 min at 65ºC. Restriction digested products were checked by 1% of agarose gel

electrophoresis to ensure complete digestion before processing next step. The digestion products then were cleaned by using a Performa® DTR 96-well purification plate (EdgeBio, Gaithersburg, MD) with manufacturer's instructions and evaporated samples. Each dried samples were reconstituted with 0.15 µl of GeneScan™ 500 LIZ™ Size Standard (Applied Biosystems) and 9.85 µl of deionized Formamide (Sigma-Aldrich). All the samples were then run in an ABI 3730 DNA Analyzer system (Applied Biosystems) to determine sizes of T-RFs from each soil sample.

Data from electropherograms was analyzed using Genemapper software version 3.5 (Applied Biosystems), a baseline threshold of 50 fluorescence units was used as an initial screen to eliminate low intensity peaks likely resulting from noise. T-RFs less than 50 base pairs (bp) and greater than 450 bp were eliminated from all datasets. Alignment of peaks between samples (also called 'binning') is required to account for T-RF drift (improperly sized T-RFs due to differences in fragment migration and purine content (Kaplan and Kitts, 2003). Raw peak height and raw peak area (before alignment) in the dataset were relativized between samples as a function of total fluorescent intensity to account for arbitrary differences in fluorescent intensity between samples. In order to avoid possible errors, relativized peak height was calculated by dividing each raw peak height by the cumulative peak height of that sample (Blackwood *et al.*, 2003). This is analogous to making each peak height a percentage of the total peak height of a sample. Likewise, relativized peak area was calculated by dividing each raw peak area by the cumulative peak area of that sample. Therefore, relativized peak height and area were applied as the TRFLP dataset of this research.

AMMI analysis of nifH DNA T-RFLP data

The web-based application T-REX (http://trex.biohpc.org) (Culman *et al.*, 2009) was used to perform T-RFs alignment across different samples and to filter noise. While fluorescence intensity was used as the basis of filtering noise the final data matrix was constructed with respect to T-RF peak presence/absence information only. The data matrix included 'environments' and T-RFs. Each 'environment' contained information on treatment identity and time of sampling. The AMMI (Additive Main effects and Multiplicative Interaction) model (Gauch, 1982) was originally designed for studying genotype response patterns across different environmental conditions. In this study, T-RFs were used as the response variable in the five agricultural treatments (with 4 replicate in each treatments). The AMMI was used to understand the responses *nifH* TRFLP pattern to different agricultural management practices represented by agricultural treatments at different time points. AMMI model uses ANOVA to first partition the variation into main effects and interactions, and then applies PCA to the interactions to create interaction principal components (IPCAs). Bi-plot of IPCAs are used for evaluating differential responses of T-RFs to the treatments.

Statistical analyses for soil characteristics in long-term agricultural treatments

Analysis of variance (ANOVA) was used to analyze the impacts of long-term agricultural treatments on soil characteristics and N-fixation. ANOVA models included one-way analysis of variance to evaluate the differences between control (NC) and agricultural sites, and 2 x 2 factorial analysis of variance to evaluate the effects of tillage and biomass retention among the agricultural sites. Statistical tests were performed using JMP 7.0 and StatView v5.0.1 (SAS Institute Inc., Cary, NC, USA).

Regression model analysis

A distance matrix was generated from the *nifH* T-RFLP data to capture differences between diazotroph community compositions in samples from different treatments at different times. The R package (vegan and LABdsv) was used to calculate the Jaccard similarity index between sample T-RF patterns. Variation in the *nifH* T-RFLP distance matrix was examined through principal coordinate analysis (PCoA) and the eigenvectors generated from this analysis were used as regressors in model construction.

Stepwise regression was performed using forward and backward procedures and collinear variables were removed so that both models produced identical results. In order to examine the possible effecters for N-fixation in soil, we used the stepwise regression model and general linear regression model (GLM) to evaluate the possibility of impacts of long-term agricultural managements, soil characteristics, and diazotrophic community composition. Student *t*-tests and ANOVA were performed for screening all predictor variables to avoid the co-relation between any predictors. The stepwise regression model was used to pick a suitable subset of important or useful variables. JMP 7.0 (SAS Institute Inc., Cary, NC, USA) was used to evaluate GLM models (Freund *et al.*, 2003). VIF (variance inflation factors) are used to evaluate the collinearity of predictor variables. The GLM model assumes a VIF of less than $1(1-R^2)$. Model VIF was calculated as $1(1-Ri^2)$, with i representing the number of independent predictor variables in each model. Model VIF exceeding $1(1-R^2)$ was used to detect and remove collinear variables from regression models (Freund *et al.*, 2003).

Results

Impacts of long-term agricultural management on soil characteristics.

From previous results, biomass and tillage both showed influences on soil characteristics at the Chazy site (Hsu and Buckley, 2009; Moebius-Clune *et al.*, 2008). However, these previous studies each relied on a single time point to reach their conclusions. In this study, soil samples were collected at different time points over a period of 2 years to account for temporal variation in the analysis of treatment effects. The purpose of this sampling was not to capture temporal variation but rather to ensure that observations of treatment effects are robust with respect to time. No clear temporal trends were observed between treatments with respect to season or sampling time. Autocorrelation between the same fields sampled at adjacent times was not observed (likely due to the low temporal density of sampling), thus, time was not treated as a main effect in subsequent analyses. The main effect of tillage was significant for 16 out of 18 soil characteristics (not including N-fixation and DNA content). In contrast, total C, Mg, P, K, Al, C/P and OM content were the only soil characteristics that were significantly impacted by the main effect of biomass treatment (Tables 3-1, 3-2 and 3-3). Significant interactions between tillage and biomass treatment, however, were observed for 7 out of 18 soil characteristics (Tables 3-1, 3-2 and 3-3). Most of extractable trace metals of NC were also significant different from agricultural sites with the exception of Mn and Zn (Table 3-3 and Table 3-4). These results largely confirm the conclusions drawn from previous analyses. Results also showed that the time had impacts on 17 out of 18 soil characteristics characteristics (Table 3-4). Only moisture and Zn were significantly affected by interaction between treatment and time.

Table 3-2. Soil and site characteristics of the long-term tillage experiment in Chazy, NY. Measured in 5 time points.

Treatment		*OM* $mg\ kg^{-1}$	*pH*	*P* $mg\ kg^{-1}$	*K* $mg\ kg^{-1}$	*Mg* $mg\ kg^{-1}$	*Ca* $g\ kg^{-1}$	*Fe* $mg\ kg^{-1}$	*Al* $mg\ kg^{-1}$	*Mn* $mg\ kg^{-1}$	*Zn* $mg\ kg^{-1}$	*Cu* $mg\ kg^{-1}$	*NO_3* $mg\ kg^{-1}$
NC	*2005/11/01*	71±4.2	6.5±0.3	1.3±0.7	80.4±32.8	222.9±24.1	2.3±0.2	3±1.9	13±7.9	15.2±1.5	1.6±0.4	1.4±0.9	17±6.8
	2006/05/24	59±3.3	8±0.1	2±0.5	56.3±8.6	182±19.4	2.2±0.2	5.1±3	20±9.9	19.3±2.2	0.7±0.2	0.9±0.3	11.8±1.4
	2006/08/10	72.7±2.4	7.6±0.5	1.8±0.9	65±12.9	211.3±34.4	2.2±0.3	4.4±1.6	13.2±5.8	9.6±1.7	0.9±0.3	2.2±0.9	21.5±1.9
	2006/09/24	74.6±4.9	8±0	1.3±0.9	53.8±7.9	204.6±33.8	2.2±0.3	3.9±1.4	15±5.7	11.8±2.1	0.7±0.3	2±0.9	14.3±2.4
	2006/11/05	73.2±4.2	7.7±0.2	1.5±0.7	48.3±6	201.3±29	2.1±0.2	4.4±1.9	17.4±9.9	11.2±2.2	1.7±0.8	3.8±1.7	12.6±3
T1	*2005/11/01*	34.3±3.6	6.4±0.3	8.9±1	42±6.6	161.8±29.1	4.5±1.1	1.1±0.7	6.2±2.3	14.4±5.2	0.9±0.1	3.9±2.2	0±0
	2006/05/24	28.1±6	7.7±0.1	9±1.8	43.3±3.3	129.5±35.1	4±1.2	2.3±0.9	12.7±2.2	17.2±5	0.2±0.1	1.5±0.7	1.3±2.7
	2006/08/10	36.4±5.4	7±0.4	12.7±2.1	39.8±7.5	143.8±34.5	4±1.4	1±0.3	7.6±1.9	10.5±3.9	0.3±0.1	2.8±0.9	41.9±34.3
	2006/09/24	37.2±5.1	7.6±0.1	10.3±1.9	30±3.6	148.3±30	4±1.1	1.1±0.4	8.1±1.5	13.5±2.6	0.2±0.1	2.6±0.7	20.9±24.6
	2006/11/05	36.4±4.6	7.2±0.3	9.5±1.8	32±9.5	138.6±32.8	4.2±1	1.8±0.9	10±2.3	12.5±6.2	0.2±0.1	3.3±0.5	6.7±8
T2	*2005/11/01*	50±9.9	6.6±0.3	10.5±1.2	38.3±5.5	179.1±42.1	3.6±1.1	0.6±0.3	4.9±1.3	10.3±1	0.9±0.3	2.9±0.3	5.2±4
	2006/05/24	41.7±7.1	7.7±0.1	14.9±9.9	54.3±5.9	151.9±28.6	3±1	1.4±0.1	10.5±1.5	14.5±0.3	0.3±0.3	1±0.6	9.3±4.4
	2006/08/10	51.3±7.1	7.1±0.4	17.2±10.4	52.5±8.5	169±22.4	2.8±0.9	1.1±0.4	8±2.8	7.5±0.6	0.4±0.2	2.1±0.2	17.5±10.8
	2006/09/24	49±6.4	7.8±0.1	9.7±1.7	31±3.4	154.7±32.2	3.2±1.2	1±0.3	7.5±1.2	9.2±0.2	0.3±0.2	2.1±0.9	9.9±8.1
	2006/11/05	49.2±10.1	7.3±0.3	9±1.9	29.8±6.9	147.1±17.3	3.1±1.1	1.1±0.3	7.9±1.2	9.4±0.4	0.3±0.3	2.5±0.8	3.1±6.2
T3	*2005/11/01*	42.1±9.3	6.5±0.3	12.6±3.8	54.1±13.3	165.2±16.2	3.9±0.3	0.7±0.8	5.7±0.6	14.8±2.2	1.1±0.2	3.2±0.7	0±0
	2006/05/24	31.9±5.8	7.9±0	9.9±2.6	56.8±6.4	138.5±20.4	3.7±0.8	2.5±1.2	12.3±2.8	18.5±5	0.1±0.1	1.3±0.4	2.9±3.4
	2006/08/10	39.1±3.9	7.7±0.3	11.1±3.9	53.3±15.8	154.1±15.4	3.7±0.6	1.5±0.9	7.4±1.9	11.6±2.9	0.3±0.1	2.2±0.3	21.1±12.6
	2006/09/24	41.7±4.8	8±0.1	8.9±1.5	41.3±12.7	143.9±27.2	3.7±0.8	1.3±0.6	8.6±2.1	12.6±3.3	0.2±0.2	2.3±0.9	12.5±14.3
	2006/11/05	38.9±6.2	7.7±0.1	8.5±2.3	46.5±7.4	147.8±26.4	3.7±0.8	2±0.9	10.1±2.3	13.6±4.3	0.3±0.1	3.3±0.6	2.7±3.2
T4	*2005/11/01*	57.8±8.2	6.6±0.3	13.2±3.3	72.4±8	238.9±31.6	3.4±0.8	0.8±0.4	3.6±0.3	10.6±1.3	1.1±0.3	3±0.7	1.3±2.5
	2006/05/24	49.2±8.4	8.1±0	16.8±10.6	81.5±17.7	199.6±25.2	3±0.7	1.8±0.2	8.3±2.1	16.4±1.6	0.3±0.2	1±0.6	4.9±3.8
	2006/08/10	57.5±7.6	7.8±0.3	26.3±9.7	70.5±12.4	212±15.9	3.2±0.6	0.9±0.3	5.4±1.8	8.6±0.4	0.3±0.1	2±1	26.4±10.7
	2006/09/24	57±6.1	8.1±0.1	14.6±2.9	61±23.8	212.5±26.3	3.1±0.5	0.9±0.2	4.6±0.7	10.4±0.2	0.3±0.1	2±0.6	7.4±2
	2006/11/05	56.1±8.2	7.8±0.3	18.4±12.5	58.8±14.5	201±20.5	3.1±0.7	1.5±0.5	6.3±0.7	10.6±1.1	0.3±0.2	2.8±0.5	1.5±3

Variation in N-fixation rates over time.

Temporal variation in N-fixation rates was large, and significant interactions were observed between sampling time and biomass and tillage treatments (Figures 3-1 and 3-2). The main effect of biomass retention was significant cross time for N-fixation rates though the main effect of tillage was not significant nor was the interation between tillage and biomass (Table 3-3). The main effect differences observed between N-fixation rates in the agricultural site and the NC control site were also significant (Table 3-4). This effect was also observed to interact as a function of time (Figure 3-1). When N-fixation rates were analyzed within time, interactions between the tillage and biomass treatments were observed within November 2005 (p = 0.0031)

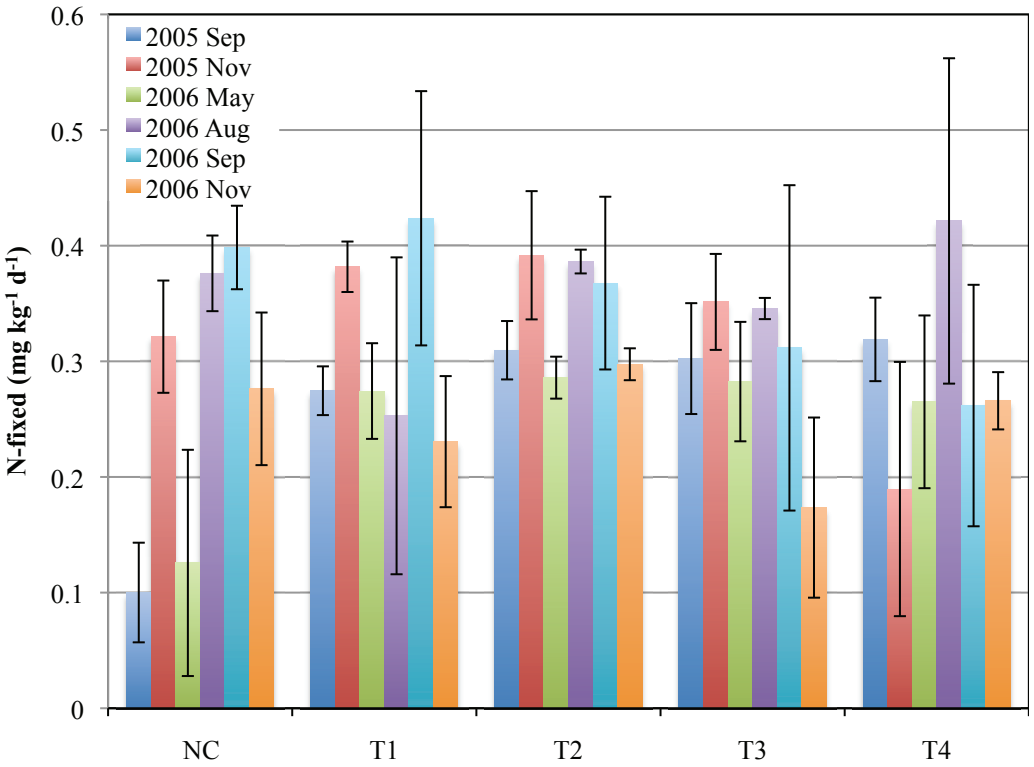

Figure 3-1. N-fixation rates in each treatment during different time points.

Table 3-3. Results from ANOVA examining effects of tillage and biomass on soil characteristics presented in table 1 and table 2 (excluding the NC sites)

	N-fixation $mg\ g^{-1}\ day^{-1}$	*DNA* $mg\ g^{-1}$	*Total C* $g\ kg^{-1}$	*Total N* $g\ kg^{-1}$	*Moisture* (%)	*OM* $mg\ kg^{-1}$	NO_3 $mg\ kg^{-1}$	*C/N*	*C/P*	*N/P*
Tillage										
Mean Square	0.004	76.2439	1183.73	11.142	291.487	4663.32	108.466	10.742	21.489	0.199
F-value	0.899	10.251	87.439	156.035	33.7985	96.470	0.8210	17.556	89.411	139.695
p-value	0.3463	0.0020	<0.0001	<0.0001	<.0001	<0.0001	0.3685	<0.0001	<0.0001	<0.0001
Biomass										
Mean Square	0.0249	71.945	92.554	0.214	30.312	665.852	247.320	2.467	1.162	0.001
F-value	5.502	9.673	6.837	2.994	3.515	13.7745	1.872	4.032	4.836	0.952
p-value	0.0218	0.0027	0.0109	0.0878	0.0649	0.0005	0.1764	0.0484	0.0317	0.3331
Tillage X Biomass										
Mean Square	0.010	11.041	92.121	0.125	3.886	46.541	158.256	8.099	1.269	0.001
F-value	2.213	1.484	6.805	1,745	0.451	0.9628	1.198	13.236	5.281	0.053
p-value	0.1412	0.2271	0.0110	0.1907	0.5042	0.3304	0.2781	0.0005	0.0251	0.4441
Time										
Mean Square	0.028	186.755	60.626	0.255	184.278	216.505	1711.93	2.991	1.554	0.008
F-value	6.215	25.108	4.478	3.566	21.367	4.479	12.957	4.888	6.466	5.387
p-value	<.0001	<.0001	0.0013	0.0062	<0.0001	0.0031	<.0001	0.0007	0.0002	0.0009
Time X Tillage										
Mean Square	0.020	9.754	27.833	0.143	7.299	5.081	168.463	0.513	0.731	0.004
F-value	4.502	1.311	2.056	2.007	0.846	0.105	1.275	0.838	3.043	3.007
p-value	0.0013	0.2689	0.0809	0.0878	0.5215	0.9803	0.2898	0.5270	0.0238	0.0250
Time X Biomass										
Mean Square	0.020	29.595	20.869	0.049	51.873	7.283	16.813	1.652	0.436	0.001
F-value	4.518	3.979	1.542	0.682	6.015	0.151	0.127	2.700	1.814	0.647
p-value	0.0012	0.0030	0.1878	0.6388	0.0001	0.9620	0.9720	0.0272	0.1380	0.6313
Time X Tillage X Biomass										
Mean Square	0.005	12.335	1.305	0.001	3.641	2.914	204.039	0.394	0.019	0.000
F-value	1.089	1.658	0.096	0.019	0.422	0.060	1.544	0.644	0.078	0.014
p-value	0.3740	0.1558	0.9925	0.9998	0.8318	0.9931	0.2009	0.6673	0.9887	0.9996

Table 3-3 (continues). Results from ANOVA examining effects of tillage and biomass on soil characteristics presented in table 1 and table 2 (excluding the NC sites).

	pH	*P* $mg\ kg^{-1}$	*K* $mg\ kg^{-1}$	*Mg* $mg\ kg^{-1}$	*Ca* $g\ kg^{-1}$	*Fe* $mg\ kg^{-1}$	*Al* $mg\ kg^{-1}$	*Mn* $mg\ kg^{-1}$	*Zn* $mg\ kg^{-1}$	*Cu* $mg\ kg^{-1}$
Tillage										
Mean Square	3.333	483.793	2469.75	31058.5	11883247	3.241	95.873	201.864	0.091	4.488
F-value	60.079	14.7776	20.551	41.320	14.005	8.914	28.898	21.657	2882	6.963
p-value	<0.0001	0.0003	<0.0001	<0.0001	0.0004	0.0127	<0.0001	<0.0001	0.0948	0.0106
Biomass										
Mean Square	0.070	161.103	8252.86	16764.2	758311	0.348	25.309	14.777	0.038	0.467
F-value	1.266	4.921	68.675	22.303	0.894	0.954	7.629	1.585	01.200	0.725
p-value	0.2651	0.0303	<0.0001	<0.0001	0.3483	0.3325	0.0076	0.2129	0.2776	0.3978
Tillage X Biomass										
Mean Square	0.051	150.742	1080.58	10995.97	702143	0.005	19.895	1.387	0.001	0.782
F-value	0.928	4.604	8.992	14.629	0.8275	0.013	5.997	0.149	0.047	1.214
p-value	0.3391	0.0360	0.0039	0.0003	0.3666	0.9115	0.0173	0.7011	0.8285	0.270
Time										
Mean Square	0.927	97.573	999.129	2398.13	412332	3.525	74.915	111.487	1.747	10.024
F-value	16.705	2.980	8.314	3.1905	0.4860	9.680	22.581	11.961	55.589	15.554
p-value	<0.0001	0.0260	<0.0001	0.0192	0.7460	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Time X Tillage										
Mean Square	0.0504	6.193	113.191	123.67	33006	0.300	3.553	1.525	0.010	0.046
F-value	0.909	1.439	0.942	0.1645	0.0389	0.824	1.071	0.164	0.305	0.072
p-value	0.4645	0.2324	0.4461	0.9555	0.9970	0.5149	0.3788	0.9560	0.8735	0.9903
Time X Biomass										
Mean Square	0.015	47.097	30.577	24.84	105393	0.111	0.294	1.451	0.056	0.154
F-value	0.269	0.189	0.254	0.0330	0.1242	0.306	0.089	0.156	1.792	0.239
p-value	0.8971	0.9432	0.9058	0.9978	0.9732	0.8732	0.9857	0.9597	0.1423	0.9151
Time X Tillage X Biomass										
Mean Square	0.008	28.847	43.685	151.27	28021	0.251	0.870	0.767	0.000	0.053
F-value	0.146	0.881	0.364	0.2012	0.0330	0.691	0.262	0.082	0.028	0.082
p-value	0.9643	0.4808	0.8336	0.9367	0.9978	0.6013	0.9010	0.9875	0.9985	0.9877

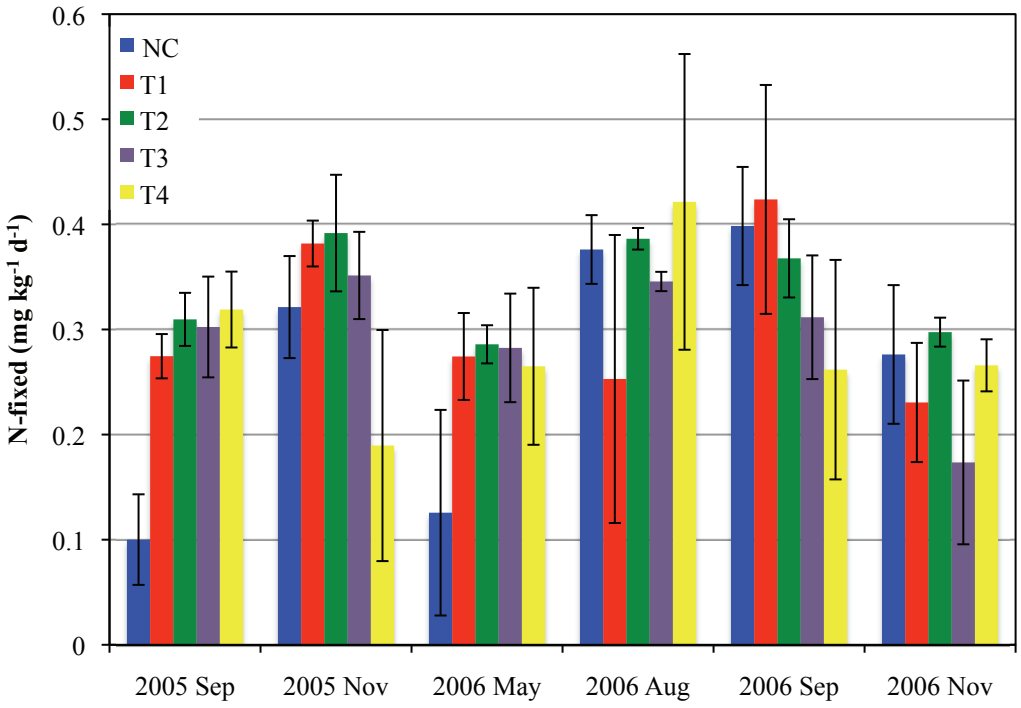

Figure 3-2. N-fixation rate of all treatments in different time points.

and November 2006 ($p = 0.0249$). N-fixation was also observed to differ significantly between the agricultural and control sites (NC) in both September 2005 ($p < 0.0001$) and May 2005 ($p = 0.0002$) (Figure 3-2). The results suggest that complex interactions govern the temporal response of N-fixation rates.

Variation of the diazotrophic community over time

A total 229 *nifH* T-RFs were observed across the 30 different sample types (5 treatments sampled at 6 times, TRFLP data from 4 replicate fields were used to represent each sample type). AMMI analysis of the data resulted in 4 IPCAs collectively explaining 51.1% of the variation in the community (Table 3-5, Table 3-6). Each of the 4 IPCAs captured meaningful variation in the diazotrophic community

Table 3-4. Results from ANOVA examining effects of agricultural managements and control sites on soil characteristics presented in table 1 and table 2.

	N-fixation $mg\ g^{-1}\ day^{-1}$	*DNA* $mg\ g^{-1}$	*Total C* $g\ kg^{-1}$	*Total N* $g\ kg^{-1}$	*Moisture* (%)	*OM* $mg\ kg^{-1}$	NO_3 $mg\ kg^{-1}$	*C/N*	*C/P*	*N/P*
Treatments Vs NC										
Mean Square	0.0317	34.981	2251.06	25.494	588.292	10694.9	500.962	19.451	74.419	0.779
F-value	5.170	2.783	89.936	151.757	48.120	112.369	4.420	26.125	146.639	219.052
p-value	0.0250	0.0982	<0.0001	<0.0001	<0.0001	<0.0001	0.0383	<0.0001	<0.0001	<0.0001
Time										
Mean Square	0.076	42.208	40.359	0.355	291.445	316.49	589.765	1.277	1.075	0.008
F-value	12.442	3.358	1.612	2.115	23.839	3.325	5.203	1.715	2.119	2.287
p-value	<0.0001	0.0074	0.1628	0.0691	<0.0001	0.0138	0.0008	0.1373	0.0850	0.0661
Treatments Vs NC X Time										
Mean Square	0.038	40.538	6.422	0.043	56.411	26.67	193.913	0.993	0.154	0.000
F-value	6.124	3.225	0.257	0.254	4.614	0.280	1.711	1.334	0.303	0.133
p-value	<0.0001	0.0094	0.9357	0.9372	0.0007	0.8901	0.1545	0.2554	0.8753	0.9698
	pH	*P* $mg\ kg^{-1}$	*K* $mg\ kg^{-1}$	*Mg* $mg\ kg^{-1}$	*Ca* $g\ kg^{-1}$	*Fe* $mg\ kg^{-1}$	*Al* $mg\ kg^{-1}$	*Mn* $mg\ kg^{-1}$	*Zn* $mg\ kg^{-1}$	*Cu* $mg\ kg^{-1}$
Treatments VS NC										
Mean Square	22.662	1939.52	2036.54	22567.6	28914072	129.787	1009.252	19.059	7.641	1.839
F-value	238.261	56.351	7.635	17.287	39.453	130.773	68.041	2.016	138.184	2.657
p-value	<0.0001	<0.0001	0.0069	<0.0001	<0.0001	<0.0001	<0.0001	0.1591	<0.0001	0.1066
Time										
Mean Square	0.327	22.834	987.870	2276.79	169656	4.791	83.107	134.376	1.734	8.807
F-value	3.434	0.663	3.704	1.744	0.232	4.827	5.603	14.215	31.355	12.727
p-value	0.0117	0.6190	0.0077	0.1472	0.9200	0.0014	0.0005	<0.0001	<0.0001	<0.0001
Treatments Vs NC X Time										
Mean Square	0.108	16.788	418.265	126.64	40044	0.589	4.318	6.921	0.495	2.980
F-value	1.137	0.488	1.568	0.097	0.055	0.594	0.291	0.732	8.957	4.306
p-value	0.3443	0.7447	0.1897	0.9832	0.9943	0.6681	0.8831	0.5724	<0.0001	0.0031

with respect to sample type. The greatest amount of variation in the diazotrophic community was due to time as captured by IPCA 1 (Figure 3-3). The bi-plot of IPCA1 and IPCA2 was able to cluster sample types across time (Figure 3-3). It can also

Table 3-5. Percent of Predicted Interaction Signal Variation Captured in IPCA.

Source	*Percent Variation*	*Cummulative Percent*
IPCA1	23.35%	23.35%
IPCA2	12.94%	36.29%
IPCA3	7.82%	44.11%
IPCA4	6.99%	51.10%

Table 3-6. ANOVA examining for AMMI model.

Source	*df*	*Sum of Saure*	*Mean Suare*
Total	6869	3598870.542	523.929
t-RFs	228	934349.709	8483.990
Env	29	69476.437	2395.739
t-RF X Evns	6612	1595044.396	241.235
IPCA1	256	372479.796	1454.999
IPCA2	254	206326.856	812.310
IPCA3	252	124747.257	495.029
IPCA4	250	111569.079	446.276
Residual	5600	779921.406	139.272

clearly be seen that the effect of time on the diazotrophic community interacts between the agricultural treatments and the control site (NC). IPCA 2 captures variation in the diazotroph community that was due differences between the NC and agricultural sites and this difference was significant (Mann-Whitney IU-Test, $p < 0.001$). When only the agricultural sites are considered it can clearly be seen the IPCA 1 clusters sites by time with the general progression September 2006 & May 2006 → November 2005 & November 2006 → September 2005 & August 2006 (Figure 3-3). The same general trend with respect to IPCA 1 can also be seen when only the NC sites are considered. The data is not sufficient to infer or determine the reason that different times cluster together, however, what the data does show is that the diazotrophic community varies

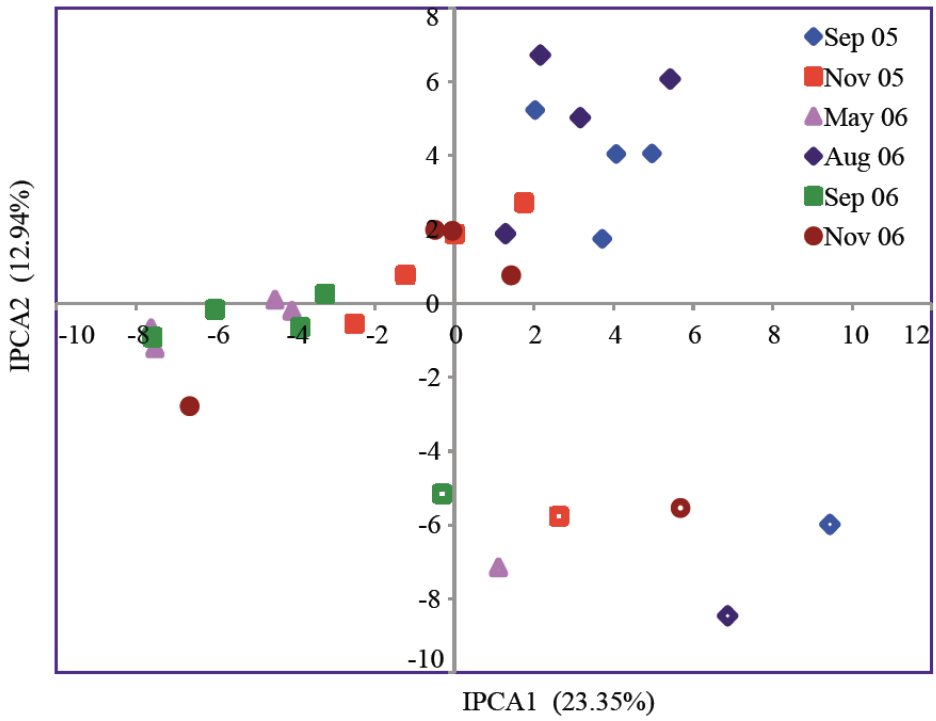

Figure 3-3. AMMI model representing the temporal variation in diazotrophic community structure. Dark grey cycles include only the agricultural treatments (T1 through T4). Yellow cycles encompass all points including site NC. Filled symbols represent agricultural treatments (T1 through T4) while hollow symbols represent NC.

significantly with time and that time is the dominant factor responsible for variation in the diazotrophic community across the sites we examined.

Despite the large temporal signal in the data, AMMI was also able to capture variation in diazotrophic community composition due to biomass and tillage management. Variation in the diazotrophic community that was caused by tillage was captured by IPCA 3 as revealed by clustering of samples associated with tilled (T1 and T3) or no till sites (T2 and T4) (Figure 3-4). Differences observed in IPCA 3 with respect to tillage management were also significant (Mann-Whitney IU-Test, $p <$ 0.001). Variation in the diazotrophic community that was caused by biomass management was captured by IPCA 4 as revealed by clustering of samples associated

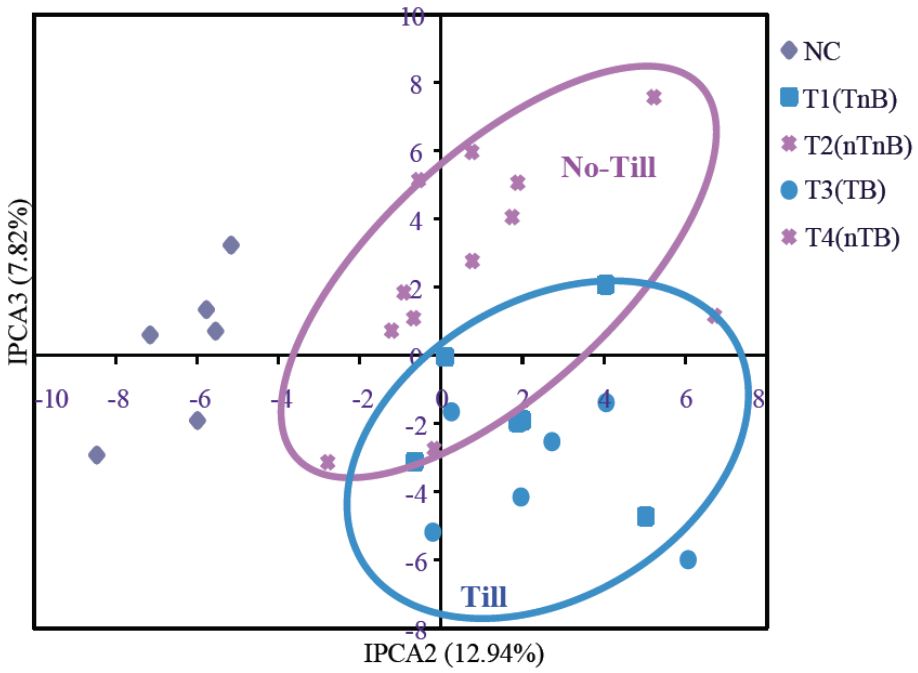

Figure 3-4. AMMI model representing the potential impact of tillage treatments on the diazotrophic community. "Till" was conventional tillage, also shown as "T" in the symbol legend. "No-till" " was no till management, also shown as "nT" in the symbol legend.

with either biomass retention (T3 and T4) or removal (T1 and T2) (Figure 3-5). Differences observed in IPCA 4 with respect to biomass management were significant (Mann-Whitney IU-Test, $p < 0.001$). As a result we can conclude that time, tillage, and biomass management have all had significant impacts on the structure of the diazotrophic community in these soils.

Evaluating the impact of soil characteristics and diazotroph community composition on variation in N-fixation rates through general linear regression models

Stepwise regression and general regression models (GLM) were used to evaluate the impacts of treatment, time, soil characteristics, and diazotrophic community composition on variation in N-fixation rates. The data used to generate regression models included time and treatment as categorical predictor variables,

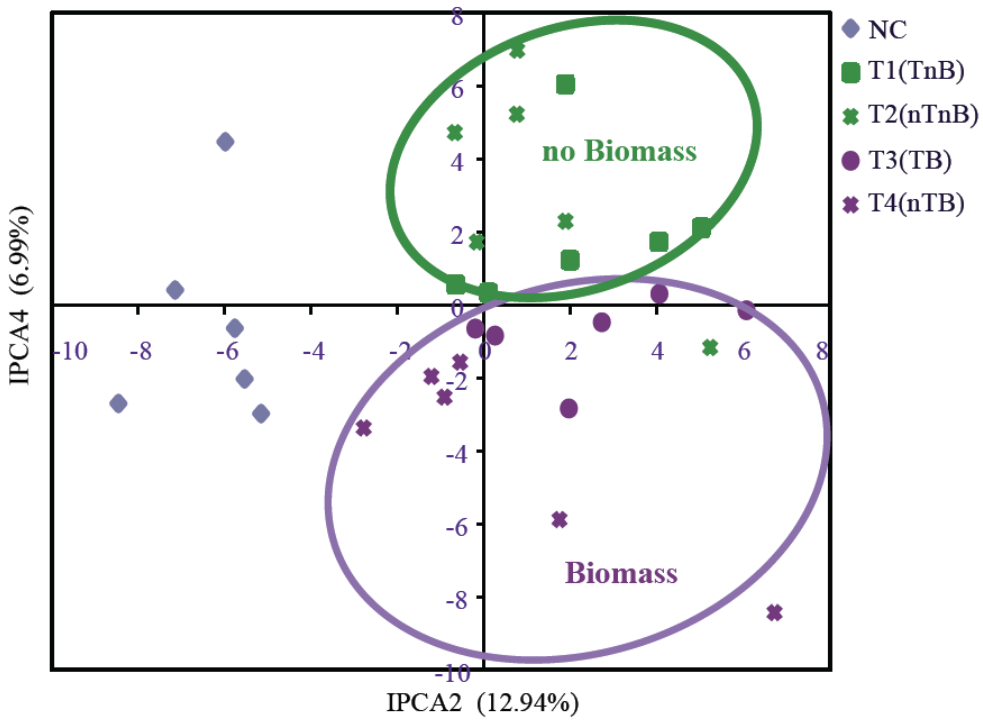

Figure 3-5. AMMI model representing the potential impact of biomass retention and removal on diazotrophic community. "Biomass" was biomass retention treatment, also shown as "B" in the symbol legend. "No Biomass" was biomass removal treatment, also shown as "nB" in the symbol legend.

values for 18 soil characteristics, and 76 eigenvalues generated from PCoA analysis of the *nifH* T-RFLP dataset were used to represent variation in the diazotroph community. ANOVA revealed co-dependences between the variables: total C, total N, % of moisture, organic matter, pH, C/N, ratio, C/P ratio and N/P ratio (all significant at $p < 0.05$). Following removal of co-dependent variables, stepwise regression was used for finding probable predictor variables (values with $p < 0.05$ were retained in the model). Two full stepwise models were constructed using N-fixation rate as a dependent variable: the first model evaluated that ability of all factors including treatment and time to predict N-fixation rates, while the second model only evaluated the ability of soil characteristics and diazotroph community structure to explain N-fixation rates.

Following removal of co-dependent variables, the first stepwise model evaluated total C, trace metal abundance, all PCoA eigenvalues, time and treatment as regressors. A total of 17 predictor variables were found to explain significant variation in N-fixation rates by the model building procedure (Tables 3-7 & 3-8). The VIF of

Table 3-7. ANOVA for GLM of N-fixation Vs. all predictor variables.

Source	*df*	*Sum of Saure*	*Mean Suare*	*F ratio*	*p-value*
Model	17	0.669	7.304	10.845	<0.00001
Error	82	0.298	183.148		
C. total	99	0.966			

the model was 3.256, which was larger than every VIF of predictor variables, indicating a lack of colinearity between the independent variables. The model explained significant variation in N-fixation rates (ANOVA, $p < 0.0001$, Table 3-7) with an $R^2 = 0.69$ (Figure 3-6). Time was the most important factor in the model

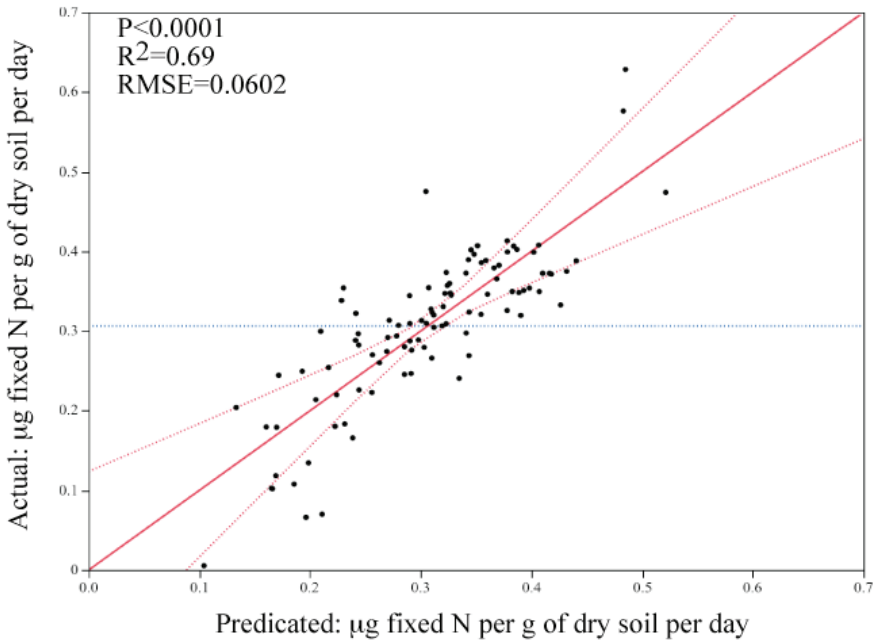

Figure 3-6. General Linear Regression Model for N-Fixation with time, treatment, diazotrophic community and soil characteristics as factors. RMSE: root mean square error; Solid red line: line of fit; dashed red line: 0.05 significance curves; dashed blue line: mean of response = 0.306.

Table 3-8. List of variables for GLM of N-fixation Vs. treatment, time, soil characteristics and diazotrophic community (represented by eigenvalues from TRFLP analysis).

Parameter Estimates	*Estimate*	*Std Error*	*t Ratio*	*Prob>|t|*	*Sum of Squares*	*F Ratio*	*V.I.F.*
Intercept	0.0606	0.0326	1.86	0.0662			
treatment{T4&T3-NC&T1}	-0.0388	0.0082	-4.73	<.0001	0.081	22.367	1.483
treatment{T4-T3}	-0.0603	0.0125	-4.84	<.0001	0.085	23.386	1.712
Time{2006MAY&2006NOV-2005NOV&2006SEP&2006AUG}	-0.0553	0.0066	-8.38	<.0001	0.255	70.215	1.151
Time{2006MAY-2006NOV}	0.0235	0.0106	2.22	0.0289	0.018	4.948	1.232
Total C mg/g	0.0081	0.0012	6.55	<.0001	0.156	42.930	1.868
P mg/Kg	0.0050	0.0011	4.47	<.0001	0.072	19.975	1.840
Eigenvalue 02	0.2101	0.0419	5.02	<.0001	0.091	25.153	1.331
Eigenvalue 07	-0.1289	0.0586	-2.20	0.0308	0.018	4.830	1.070
Eigenvalue 10	-0.1498	0.0751	-1.99	0.0495	0.014	3.976	1.143
Eigenvalue 12	0.2135	0.0757	2.82	0.006	0.029	7.945	1.110
Eigenvalue 23	-0.3624	0.1030	-3.52	0.0007	0.045	12.368	1.088
Eigenvalue 28	-0.5320	0.1230	-4.32	<.0001	0.068	18.692	1.168
Eigenvalue 35	-0.4306	0.1515	-2.84	0.0057	0.029	8.077	1.148
Eigenvalue 46	-0.3616	0.1810	-2.00	0.0490	0.014	3.993	1.049
Eigenvalue 49	0.7287	0.1942	3.75	0.0003	0.051	14.080	1.081
Eigenvalue 50	0.9186	0.2159	4.25	<.0001	0.066	18.098	1.132
Eigenvalue 56	0.6062	0.2403	2.52	0.0136	0.023	6.361	1.088

explaining 24.64% of the variation in N-fixation rates ($R^2 = 0.2464$, F ration = 7.766, $p < 0.0001$). Variation in diazotrophic community structure (as represented by 8 distinct eigenvalues from the *nifH* TRFLP data) explained 23.4% of the variation in N-fixation rates ($R^2 = 0.234$, F ration = 2.4459, $p = 0.0103$). Soil characteristics ($R^2 = 0.026$, F ration = 1.312, $p = 0.2740$) and experimental treatment ($R^2 = 0.009$, F ration = 1.312, $p = 0.2740$) were each on their own unable to explain significant variation in N-fixation rates in this model.

The second model was constructed as above except that treatment and time were excluded as categorical variables to solely assess the ability of soil characteristics and diazotrophic community structure to explain N-fixation rates. A total of 11 predictor variables were found to explain significant variation in N-fixation rates (Tables 3-9 & 3-10). The VIF of the model was 1.887, which was larger than every VIF of predictor variables, indicating a lack of colinearity between the independent

Table 3-9. ANOVA for GLM of N-fixation Vs. soil characteristics and diazotrophic community.

Source	*df*	*Sum of Saure*	*Mean Suare*	*F ratio*	*p-value*
Model	11	0.454	0.041	7.0914	<0.0001
Error	88	0.512	0.006		
C. total	99	0.966			

variables. The model explained significant variation in N-fixation rates (ANOVA, $p < 0.0001$, Table 3-9) with an $R^2 = 0.47$ (Figure 3-7). Diazotroph community structure (as represented by 8 distinct eigenvalues from the *nifH* TRFLP data) was the most important factor in the model explaining 30.3% of the variation in N-fixation rates ($R^2 = 0.303$, F ration = 4.905, $p < 0.0001$). Variation in soil characteristics explained only 12.34% of the variation in N-fixation rates ($R^2 = 23.34$, F ration = 4.506, $p = 0.0.005$).

Table 3-10. List of variables for GLM of N-fixation Vs. soil characteristics and diazotrophic community (represented by eigenvalues from TRFLP analysis).

Parameter Estimates	*Estimate*	*Std Error*	*t Ratio*	*Prob>|t|*	*Sum of Squares*	*F Ratio*	*V.I.F.*
Intercept	0.250	0.032	7.92	<.0001			
Total C mg/g	0.003	0.0018	2.51	0.0137	0.039	6.3220	1.481
Aluminum mg/Kg	-0.005	0.001	-3.08	0.0028	0.055	9.4684	1.435
NO3 mg/Kg	0.002	0.001	3.57	0.0006	0.074	12.7305	1.179
Eigenvalue 02	0.154	0.052	3.01	0.003	0.053	9.0729	1.241
Eigenvalue 04	0.152	0.06	2.49	0.015	0.036	6.2134	1.229
Eigenvalue 05	-0.159	0.065	-2.45	0.016	0.035	6.0251	1.194
Eigenvalue 23	-0.440	0.127	-3.47	0.0008	0.070	12.0650	1.024
Eigenvalue 28	-0.308	0.145	-2.11	0.0373	0.026	4.4731	1.017
Eigenvalue 45	-0.646	0.216	-2.99	0.0037	0.052	8.9217	1.030
Eigenvalue 49	0.694	0.242	2.86	0.0052	0.048	8.2003	1.048
Eigenvalue 50	0.726	0.263	2.76	0.0070	0.044	7.6324	1.043

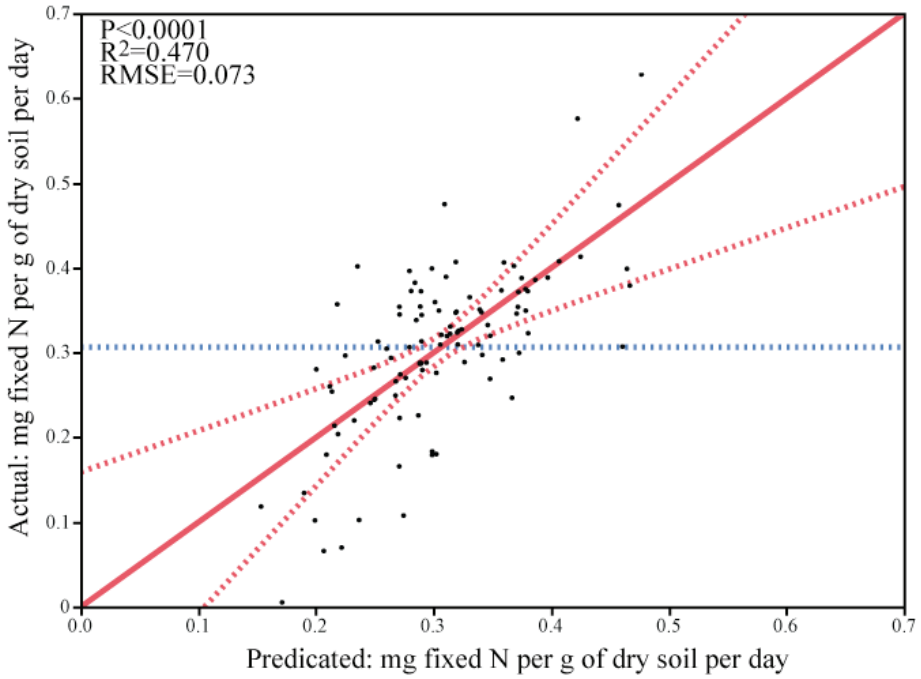

Figure 3-7 General Linear Regression Model for N-Fixation with soil characteristics and diazotrophic community as factors. RMSE: root mean square error; Solid red line: line of fit; dashed red line: 0.05 significance curves; dashed blue line: mean of response = 0.306.

Next we generated 4 separate GLM models to estimate independently the amount of variation in N-fixation rates that is explained by each of the factors: treatment, time, soil characteristics and diazotrophic community structure. All 4 GLM models were significant (Tables 3-11 to 3-13). The largest amount of variation in N-fixation rates was explained by diazotroph community structure ($R^2 = 0.315$), followed by time ($R^2 = 0.237$), soil characteristics ($R^2 = 0.126$), and treatment ($R^2 = 0.040$).

Table 3-11. ANOVA for 4 GLMs of N-fixation.

	Source	*df*	*Sum of Saure*	*Mean Suare*	*F ratio*	*p-value*
Treatments	*Model*	1	0.039	0.039	4.116	0.0452
	Error	98	0.928	0.009		
	C. total	99	0.966			
Times	*Model*	1	0.229	0.229	30.465	<0.0001
	Error	98	0.737	0.008		
	C. total	99	0.966			
Soils	*Model*	2	0.122	0.61	6.9977	0.0014
	Error	97	0.845	0.009		
	C. total	99	0.966			
Diazotrophic community	*Model*	7	0.304	0.043	6.0439	<0.0001
	Error	92	0.662	0.007		
	C. total	99	0.966			

Table 3-12. Summary of Fit for 4 GLMs of N-fixation. (Observations = 100).

Treatments vs N-fixation	*R-Square*	0.040
	R^2 Adjl	0.031
	Root Mean Square Error	0.097
	Mean of Suare	0.306
Times vs N-fixation	*R-Square*	0.237
	R^2 Adjl	0.229
	Root Mean Square Error	0.087
	Mean of Suare	0.306
Soil vs N-fixation	*R-Square*	0.126
	R^2 Adjl	0.108
	Root Mean Square Error	0.093
	Mean of Suare	0.306
Diazotrophic community vs N-fixation	*R-Square*	0.315
	R^2 Adjl	0.263
	Root Mean Square Error	0.085
	Mean of Suare	0.306

Table 3-13. Variables of different sets for 4 GLMs of N-fixation.

Parameter Estimates		*Estimate*	*Std Error*	*t Ratio*	*Prob>|t|*	*Sum of Squares*	*F Ratio*	*V.I.F.*
Treatments	*Intercept*	0.321	0.012	26.41	<.0001			
	T4&T3&NC&T1-T2	-0.025	0.012	-2.03	0.0452	0.039	4.1157	1.0
Times	*Intercept*	0.297	0.009	33.50	<.0001			
	2006MAY&2006NOV-2005NOV&2006SEP&2006AUG	-0.049	0.009	-5.52	<.0001	0.229	30.465	1.0
Soil characteristics	*Intercept*	0.355	0.038	9.28	<.000			
	Cal	0.00002	0.00001	2.02	0.0456	0.036	4.010	1.137
	Mn	-0.009	0.003	-3.65	0.0004	0.116	13.346	1.137
diazotrophic community	*Intercept*	0.303	0.009	35.28	<.0001			
	Eigenvalue 02	0.110	0.051	2.14	0.0353	0.033	4.564	1.005
	Eigenvalue 14	-0.291	0.110	-2.65	0.0096	0.050	7.004	1.017
	Eigenvalue 23	-0.394	0.140	-2.82	0.0058	0.057	7.969	1.006
	Eigenvalue 28	-0.334	0.161	-2.08	0.0403	0.031	4.325	1.005
	Eigenvalue 45	-0.560	0.238	-2.35	0.0207	0.040	5.540	1.007
	Eigenvalue 49	0.754	0.264	2.86	0.0053	0.059	8.172	1.005
	Eigenvalue 50	0.892	0.290	3.08	0.0027	0.068	9.482	1.027

Discussion

This study was conducted to follow up on observations made from samples taken in November 2005 which were described in Hsu and Buckley 2009 (Chapter 2). Temporal variation in soil processes is common and so in order to make robust analyses of treatment effects or relationships between soil processes and environmental variables it is important to make multiple observations over time. With respect to soil characteristics, the treatment effects described in previouse results were robust when observations were expanded to multiple sampling times. Most soil characteristics were impacted primarily by tillage or were impacted by interactions between tillage and biomass management (Table 3-1). The main effect of long term biomass retention was to increase levels of C, N, P, K, Mg, and OM (Table 3-1) as would be expected. This result was similar to what we have observed in Hsu and Buckley 2009 (Chpater2). Pervious research suggested that seasonal pattern of forests' soil characteristics (moisture, C, N C/N) and nutrient flushes were observe within the sampling times (Díaz-Raviña *et al.*, 1993; Pérez *et al.*, 2004). In our study, the measured soil characteristics were significant over time but interactions between treatment and time were not observed (Table 3-3 and 3-4). These results suggested that time had impacts on soil characteristics with possible relationships to seasonal patterns. N-fixation rates also varied significantly over time but despite this variation the main effect of biomass management was still significant when assessed over time. The main effect of tillage, however, was not significant across time (Table 3-3).

Certain soil characteristics might be expected to explain soil N-fixation rates. For example, increases in C (Kondo and Yasuda, 2003a; Kondo and Yasuda, 2003b) and C/N (Maheswaran and Gunatilleke, 1990; Vitousek, 1994) would be expected to stimulate N-fixation while increases in N (Compton *et al.*, 2004; Yang *et al.*, 2007) would be expected to decrease N-fixation rates. Meanwhile soil pH can possibly

impact the availability of molybdenum with lower pH values found to inhibit N-fixation (Goldberg, 2009). In simple linear regression analyses, however, there were no significant relationships observed between C, N, C/N, or pH and N-fixation. Paradoxically, soil NO_3 exhibited a positive though weak association with N-fixation rates. In previous experiments N-fertilization of sediments has actually been observed to cause stimulation of N-fixation, presumably by promoting enrichment of standing microbial biomass. In addition, Fe, Al and Mn all had weak negative associations with N-fixation rate. While significant associations were observed between soil variables and N-fixation rates these associations were generally weak.

TRFLP is a high-throughput fingerprinting method for studying microbial community composition under different conditions. TRFLP need not be only applied to the study of total bacterial or fungal SSU ribosomal DNA under different ecosystems (Braker *et al.*, 2001; Kennedy *et al.*, 2005; Meier *et al.*, 2008). Recently, TRFLP has also been used for studying functional genes such as *nifH*, *nosZ*, and *amoA* (Hallin *et al.*, 2009; Rosch and Bothe, 2005) to understand functional group composition in different ecosystems. Most of these studies were focused on the relationships between functional group composition and environmental characteristics. In our study, TRFLP analysis of *nifH* was used to evaluate the structure of the diazotroph community under different agricultural management practices. The AMMI model used to examine variation in *nifH* TRFLP profiles made it possible to visualize the interaction between the environmental factors and the diazotrophic community. Time was associated with the largest source of variation in the diazotroph community. After time, the second largest source of variation was caused by differences between the diazotroph community in the long-term agricultural sites and the never cultivated control site. Significant variation in the diazotroph community was also observed in response to tillage and biomass management. These treatment level effects on

diazotroph community structure are consistent with observations made of diazotroph community composition made in November 2005 (as described in Hsu and Buckley 2009). The fact that both diazotroph community structure and N-fixation rates varied dramatically over time while soil characteristics did not led us to perform more sophisticated analyses to investigate the degree to which these different factors are associated with soil N-fixation rates.

In order to understand how the diazotrophic community related to N-fixation, variation in N-fixation rates was examined in relation to variation in *nifH* TRFLP data and soil characteristics over time by using general linear regression models. Similar analyses have been used to examine relationships between nitrification and *amoA* in a marine study (Wuchter *et al.*, 2006). Eigenvalues from PCoA of *nifH* T-RFLP were used in regression analysis to provide continuous variables that capture significant variation in diazotroph community composition. In all regression models we constructed, diazotroph community structure explained a greater proportion of the variation in N-fixation rates than could be explained by soil characteristics. In the first stepwise model the factors of time, treatment (with NC included), total C, total P and diazotrophic community structure explained 69% of variation in N-fixation rates. The second full model excluded time and treatment as factors. This model was constructed because certain soil and community variables (most notably C, N, OM, pH, and moisture) co-varied with treatment and time and thus needed to be excluded from the model when these factors were included in the analysis. This second model explained 47% of variation in N-fixation. The 4 GLM models used only time, treatment, soil characteristics or diazotrophic community structure as predictor values in order to better isolate the impacts of these factors on N-fixation rates. In both the second stepwise model and the 4 GLM models where time was excluded as an explicit factor in the analysis, the structure of the diazotrophic community was found to be the single

largest factor explaining the variation in N-fixation rates that we observed in this study. This suggests that changes that occur in the diazotroph community over time are major drivers of N-fixation rates. The diazotroph community composition is in turn sensitive to treatment effects leading to interactions between treatment and time, which could influence their N-fixation rates. Cusack and collegues (Cusack *et al.*, 2009) studied the effects of N-fertilization on biological N fixation in tropical forests. In their study, ANOVA, analysis of covariance (ANCOVA) and Fisher's Least Significant Difference test were applied to soil characteristics and N-fixation. Their results could demonstrate that N-fixation was significantly related to moisture content and sensitived to N-deposition, however in their study they did not examine relationships between N-fixation and variation in diazotroph community composition. In this chapter, we demonstrated that changes in the structure of the diazotrophic community can explain significant variation in soil N-fixation rates. These data are consistent with and extend those presented in Chapter 2. The data show that soil characteristics such as C, N, moisture, and pH are not sufficient for understanding spatial or temporal variation in N-fixation at the field scale.

REFERENCES

Blackwood C, Marsh T, Kim S, Paul E (2003). Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. *Appl Environ Microbiol* **69:** 926 - 932.

Braker G, Ayala-del-Rio HL, Devol AH, Fesefeldt A, Tiedje JM (2001). Community structure of denitrifiers, bacteria, and archaea along redox gradients in pacific northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (nirS) and 16S rRNA genes. *Appl. Environ. Microbiol.* **67:** 1893-1901.

Brouzes R, Lasik J, Knowles R (1969). Effect of organic amendment, water content, and oxygen on incorporation of $15N_2$ by some agricultural and forest soils. *Can J Microbiol* **15:** 899-905.

Burgmann H, Widmer F, Von Sigler W, Zeyer J (2004). New molecular screening tools for analysis of free-living diazotrophs in soil. *Appl. Environ. Microbiol.* **70:** 240-247.

Burt R (2004). Soil Survey Laboatory Methods Manual. In: Burt R (ed). *Soil Survey Investigations Report No. 42 Version 4.0*. Lincoln, Nebraska U.S. Department of Agriculture, Natural Resources Conservation Service (USDA). p 700.

Cleveland CC, Townsend AR, Schimel DS, Fisher H, Howarth RW, Hedin LO *et al* (1999). Global patterns of terrestrial biological nitrogen (N2) fixation in natural ecosystems. *Global Biogeochem. Cycles* **13:** 623-645.

Compton JE, Watrud LS, Arlene Porteous L, DeGrood S (2004). Response of soil microbial biomass and community composition to chronic nitrogen additions at Harvard forest. *Forest Ecology and Management* **196:** 143-158.

Culman S, Bukowski R, Gauch H, Cadillo-Quiroz H, Buckley D (2009). T-REX: software for the processing and analysis of T-RFLP data. *BMC Bioinformatics* **10:** 171.

Cusack D, Silver W, McDowell W (2009). Biological nitrogen fixation in two tropical forests: Ecosystem-level patterns and effects of nitrogen fertilization. *Ecosystems* **12:** 1299-1315.

Díaz-Raviña M, Acea MJ, Carballas T (1993). Seasonal fluctuations in microbial populations and available nutrients in forest soils. *Biology and Fertility of Soils* **16:** 205-210.

Freund R, Littell R, Creighton L (2003). *Regression Using JMP*. SAS Press and John Wiley Sons Inc., 288pp.

Gauch HG (1982). *Multivariate analysis in community ecology.* Cambridge University Press, Cambridge.

Goldberg S (2009). Influence of soil solution salinity on molybdenum adsorption by soils. *Soil Science* **174:** 9-13 10.1097/SS.0b013e318195b827.

Hallin S, Jones CM, Schloter M, Philippot L (2009). Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. *ISME J* **3:** 597-605.

Howard JB, Rees DC (1996). Structural Basis of Biological Nitrogen Fixation. *Chemical Reviews* **96:** 2965-2982.

Howarth RW, Marino R, Lane J, Cole JJ (1988). Nitrogen fixation in freshwater, estuarine, and marine ecosystems. 1. Rates and importance. *Limnology and Oceanography* **33:** 669-687.

Hsu S-F, Buckley DH (2009). Evidence for the functional significance of diazotroph community structure in soil. *ISME J* **3:** 124-136.

Kaplan CW, Kitts CL (2003). Variation between observed and true Terminal Restriction Fragment length is dependent on true TRF length and purine content. *Journal of Microbiological Methods* **54:** 121-125.

Kennedy N, Edwards S, Clipson N (2005). Soil bacterial and fungal community structure across a range of unimproved and semi-improved upland grasslands. *Microbial Ecology* **50:** 463-473.

Kondo M, Yasuda M (2003a). Effects of Temperature, Water Regime, Light, and Soil Properties on $15N_2$ Fixation Associated with Decomposition of Organic Matter in Paddy Soils. *Japan Agricultural Research Quarterly* **37:** 113-119.

Kondo M, Yasuda M (2003b). Seasonal changes in N_2 fixation activity and N enrichment in paddy soils as affected by soil management in the northern area of Japan. *Jpn Agric Res Q* **37:** 105-111.

Leigh GJ (2002). *Nitrogen fixation at the millennium* Elsevier B.V.

Liu W, Marsh T, Cheng H, Forney L (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl. Environ. Microbiol.* **63:** 4516-4522.

Maheswaran J, Gunatilleke IAUN (1990). Nitrogenase activity in soil and litter of a tropical lowland rain forest and an adjacent fernland in Sri Lanka. *Journal of Tropical Ecology* **6:** 281-289.

Meier C, Wehrli B, van der Meer J (2008). Seasonal fluctuations of bacterial community diversity in agricultural soil and experimental validation by laboratory disturbance experiments. *Microbial Ecology* **56:** 210-222.

Moebius-Clune BN, van Es HM, Idowu OJ, Schindelbeck RR, Moebius-Clune DJ, Wolfe DW *et al* (2008). Long-term effects of harvesting maize stover and tillage on soil quality. *Soil Sci Soc Am J* **72:** 960-969.

Montoya JP, Voss M, Kahler P, Capone DG (1996). A simple, high-precision, high-sensitivity tracer assay for N_2 fixation. *Appl. Environ. Microbiol.* **62:** 986-993.

Newton WE (2008). *Nitrogen-fixing leguminous symbioses*. Springer.

Nohrstedt HO (1983). Conversion factor between acetylene reduction and nitrogen fixation in soil: effects of water content and nitrogenase activity. *Soil Biol Biochem* **15:** 275-279.

Patriquin D, Knowles R (1972). Nitrogen fixation in the rhizosphere of marine angiosperms. *Marine Biology* **16:** 49-58.

Pérez CA, Carmona MR, Aravena JC, Armesto JJ (2004). Successional changes in soil nitrogen availability, non-symbiotic nitrogen fixation and carbon/nitrogen ratios in southern Chilean forest ecosystems. *Oecologia* **140:** 617-625.

Postgate J (1998). *Nitrogen Fixation, 3rd Edition*, third edn. Cambridge University Press , Cambridge, UK.

Raymond J, Siefert JL, Staples CR, Blankenship RE (2004). The natural history of nitrogen fixation. *Mol Biol Evol* **21:** 541-554.

Rosch C, Bothe H (2005). Improved assessment of denitrifying, N_2-fixing, and total-community bacteria by terminal restriction fragment length polymorphism analysis using multiple restriction enzymes. *Appl. Environ. Microbiol.* **71:** 2026-2035.

Skujins J, Tann CC, Borjesson I (1987). Dinitrogen fixation in a montane forest sere determined by 15N2 assimilation and in situ acetylene-reduction methods. *Soil Biol Biochem* **19:** 465-471.

Steward GF, Zehr JP, Jellison R, Montoya JP, Hollibaugh JT (2004). Vertical distribution of nitrogen-fixing phylotypes in a meromictic, hypersaline lake. *Microbial Ecology* **47:** 30-40.

van Dommelen A, and , Vanderleyden J (2007). *Biology of the Nitrogen Cycle*. ELSEVIER.

Vitousek PM (1994). Potential nitrogen fixation during primary succession in Hawaii volcanoes national park. *Biotropica* **26:** 234-240.

Wuchter C, Abbas B, Coolen MJL, Herfort L, van Bleijswijk J, Timmers P *et al* (2006). Archaeal nitrification in the ocean. *Proceedings of the National Academy of Sciences* **103:** 12317-12322.

Yang X, Warren M, Zou X (2007). Fertilization responses of soil litter fauna and litter quantity, quality, and turnover in low and high elevation forests of Puerto Rico. *Applied Soil Ecology* **37:** 63-71.

Zehr JP, Jenkins BD, Short SM, Steward GF (2003). Nitrogenase gene diversity and microbial community structure: a cross-system comparison. *Environmental Microbiology* **5:** 539-554.

CHAPTER 4

EVALUATING THE EFFECTS OF AGRICULTURAL MANAGEMENT, AND DEPTH ON DIAZOTROPH COMMUNITY COMPOSITION IN SOIL

Abstract

Factors that impact the distribution of diazotrophs in soils and the diversity of their communities remain poorly characterized. In this research project, we evaluated *nifH* gene sequences in soils from two long-term agricultural experimental sites to understand impacts of soil type, soil depth, vegetation, and agricultural history on the diversity of the diazotroph community in soil. Soil type/location was a primary driver of diazotroph community composition. Both a history of agricultural management and increasing soil depth were associated with reductions in diazotroph diversity. Certain groups of diazotrophs were associated specifically with mono-cropping maize activity while others were associated with non-agricultural grassland. These observations provide insights on factors that impact the structure of diazotroph communities in soil.

Introdution

In the previous chapters we have used *nifH* clone libraries and DNA TRFLP data to study of diazotrophic community in a long term agricultural site in Chazy, Clinton, NY. (Chapter 2 and Chapter 3). Maize stover biomass retention for 35 years caused a dramatic change in the evenness of the diazotroph community, which indicated a significant effect of biomass management on diazotroph community structure. This change in evenness was primarily driven by the enrichment of a single group of dominant *nifH* sequences in response to biomass retention. However, tillage also had a significant impact on community structure. Taken together these results indicate an interaction between the effects of tillage and biomass retention on

diazotroph community structure. Also, the diazotrophic community in a control site of Chazy (which is maintained as a fallow field) was different from that found in adjacent agricultural sites. The result indicated that agricultural management had decreased the richness and evenness of the diazotrophic community. In addition, group 1A diazotrophs (Zehr *et al.*, 2003) were found to be abundant in the never cultivated grassy field and were nearly absent from the agricultural site.

A number of studies have revealed that long-term agricultural management practices have profound and lasting impacts on microbial community structure in soil. Buckley and Schmidt (Buckley and Schmidt, 2003) demonstrated that the structure of microbial communities subject to long term agricultural practices took years to respond to changes in management practice and that microbial communities in agricultural fields were dramatically different from those in fields managed historically without cultivation. Girvan et al (Girvan *et al.*, 2003) demonstrated that soil type was the key factor in determining bacterial community composition in arable soils, with geographic location and land-use practices having lesser effects. Studies of tillage impacts on microbial community structure have also demonstrated the potential for tillage to reduce microbial diversity in many studies (Allison *et al.*, 2005; Drijber *et al.*, 2000; Entry *et al.*, 2008). Residue input from crops or cover crops might be expected to stabilize soil quality and have impacts on microbial community structure (Dilly *et al.*, 2004; Karlen *et al.*, 1994). There is also evidence that diazotrophic communities and their nitrogenase activities are influenced by different cropping systems and crop varieties (Mårtensson *et al.*, 2009; Roesch *et al.*, 2008; Roesch *et al.*, 2006; Wu *et al.*, 2009).

Understanding the factors that influence diazotrophic community composition is required to develop an understanding of whether community composition has an impact on the N-fixation rate in environments. Agricultural sites can provide relatively

simple, well characterized ecosystems in which to study the functional significance of microbial community structure in soil (Reed and Martiny, 2007). In this chapter, we examine another long-term agricultural site in Caldwell Field, Ithaca, NY. Comparing the diazotrophic community structures in Caldwell Field to those at the Chazy site can provide further evidence as to the forces that impact the structure of the diazotrophic community in soil. We primarily focus on the impacts of soil depth, soil type, vegetation, and agricultural activity on diazotrophic community structure. Three components of community structure: the evenness, richness and composition of the diazotrophic community were examined.

Materials and Methods

Site description and soil sampling.

Soil samples were taken from two long-term tillage experiment fields located at the Miner Institute, Chazy, Clinton County, NY (N 44.884672, W −73.474429) and the Cornell University Caldwell Field Research Farm, Ithaca, Tompkins County, NY (N 42.450061, W−76.458782). The soil type at Chazy is Raynham silt loam and that at Caldwell field is Niagara silt loam (Buckley *et al.*, 2007; Moebius-Clune *et al.*, 2008). Raynham silt loam and Niagara silt loam soils are both mixed, active, nonacid, and mesic Aeric Epiaquepts that have poor drainage properties. They differ in that the Raynham silt loam has a coarse-silty texture while that of the Niagara silt loam is fine-silty. The A horizons of the Raynham and Niagara silt loams extend between 0 to 15 cm and 0 to 12.7 cm respectively, and both consist of dark grayish brown silt loams that are slightly acid, composed of moderate fine and medium granular structure, and are friable and with many roots. At 40-50 cm the Raynham silt loam contains Bw and Bg horizons while the Niagara silt loam contained a Bt horizon with both soils having

few roots and iron accumulation at this depth (http://soils.usda.gov/technical/classification/osd/index.html).

The sites were both managed as agricultural fields for more than 30 years, and were used for maize at the time of sampling, and each contained a control field that had never been cultivated. Two sets of plots from Chazy (as described in earlier chapters) were included in this analysis: long-term continuous maize plots managed by no-till and corn stover biomass retention (T2), and never cultivated control plots (NC) that were mowed monthly and were adjacent to and on the same soil as the agricultural plots. In addition, two sets of plots from Caldwell Field were examined: long-term agricultural plots managed with crop rotation and conventional tillage (I), and never cultivated control plots (G) that were mowed monthly and were adjacent to and on the same soil as the agricultural plots. Caldwell field I was fallow from 1996-2001, was used for alfalfa in 2002-2004, and was used for Maize from 2004 through the time of sampling in 2007. Miaze was grown at both Chazy and Caldwell.

Soil samples from Chazy NC and T2 were taken on 1 Nov 2005. Soil cores (2.5 cm diameter) were used to obtain samples at a depth of both 0-5 cm (n = 20) and 40-50 cm (n =7) from each of 4 replicate plots. The cores from each replicate plot at each depth were subsequently sieved to 2 mm and homogenized to provide aggregate samples. Caldwell G and I were sampled in a similar manner in July 2007 at a depth of both 0-5 cm (n = 7) and 40-50 cm (n =5) from each of 7 replicate plots. Samples were frozen in liquid nitrogen in the field, transported on dry ice, and stored at -80°C.

Construction of nifH clone libraries

Methods used to construct *nifH* clone libraries were described in Chapter 2 (Hsu and Buckley, 2009) and are briefly summarized in this section. Soil DNA was extracted from subsamples of 0.33 g of each soil sample using PowerSoil™ DNA

Isolation Kit (MoBio, Inc, Carlsbad, California) as per manufacturer's instructions. Three parallel DNA extractions were performed for each sample and the resulting DNA samples were pooled. DNA concentrations were determined through the Pico-Green assay (Invitrogen, Carlsbad, California) as per manufacturer's instructions. For construction of *nifH* clone libraries, DNA from the replicate plots was pooled proportionally by volume to obtain a single sample for each depth (top: 0-5 cm; deep: 40-50 cm) from each of the fields T2, NC, G, and I. Primers *nif*H-b1-112F (Burgmann *et al.*, 2004) and *nifH*623R (Steward *et al.*, 2004) were used to conduct PCR amplification of *nifH* genes. PCR reactions were conducted in 50 μl volumes containing 70 ng of template DNA, with each primer at a concentration of 0.25 μM, each dNTP at a concentration of 200 μM, 2.5 mM $MgCl_2$, 0.05% of BSA (New England Biolab), 2.5 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, California), and 1x PCR buffer (supplied with Taq enzyme). Each PCR consisted of a 95°C hold for 10 min followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 45 s at 72°C; and a final extension for 15 min at 72°C. Three PCR reactions were performed in parallel for each pooled DNA sample, these PCR products were combined and purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin), and then cloned into pCR2.1-TOPO as per manufacturer's instructions (Invitrogen, Carlsbad, California). DNA sequencing was performed using an Applied Biosystems Automated 3730 DNA Analyzer at Cornell University's Biotechnology Resource Center. The nucleotide sequences of the 546 *nifH* gene clones described in this study have been deposited in GenBank under accession numbers: XXXXX:YYYYY.

Phylogenetic analysis of nifH sequences

Phylogenetic analysis was performed as described in Chapter 2. Sequences were imported into ARB (Strunk, 1997) and aligned against a *nifH* database constructed from sequences available in Genbank that were aligned against the Pfam Fer4_NifH amino acid seed alignment (Finn *et al.*, 2006). Regions of ambiguous alignment were identified and excluded from subsequent phylogenetic analyses. Phylogenetic trees were generated by performing protein parsimony (Swofford, 1991), and maximum-likelihood analyses (Olsen *et al.*, 1994) using Phylip 3.64 (Felsenstein, 2005).

Analysis of diazotroph community structure

Diazotroph community composition was assessed using the approach described in Chapter 2 with modifications as described below. Operational taxonomic unit (OTU) classification was performed on aligned DNA sequences of 487 nucleotides in length using DOTUR (Schloss and Handelsman, 2005). An OTU cutoff of 93% similarity was used for diversity calculations since this level of nucleotide divergence in conserved genes roughly corresponds to the expected level of nucleotide divergence found between different microbial species (Konstantinidis and Tiedje, 2005). Community structure was evaluated as a function of changes in community diversity and composition as previously discussed (Schloss, 2008). The ChaoI estimator (Hughes *et al.*, 2001), Shannon index and the evenness component of the Shannon Index (Pielou's J') (Magurran, 1988) were used to evaluate both the richness and evenness component of the community. UniFrac was performed with both weighted and un-weighted data (which ignores the relative abundance of OTUs in the library). Unifrac assesses overall differences in community structure and is sensitive to changes in richness, evenness, genetic diversity, and the composition of communities

as previously described (Schloss, 2008). Guide trees for UniFrac were generated through maximum-likelihood analysis as described above. Distance matrices generated with Unifrac were used to cluster communities using UPGMA and jackknife analysis was used to evaluate to the confidence of tree nodes.

Statistical analyses

Statistical tests were performed using JMP 7.0 (SAS Institute, Inc., Cary, North Carolina). Analysis of variance (ANOVA) models included one-way ANOVA to evaluate the main effects of among the 0-5 cm soil samples of Chazy and Caldwell sites.

Results

The impact of long-term cultivation on the diversity of the diazotroph community

When assessed across soil type, a decrease in *nifH* richness and evenness was observed in sites subject to long-term agricultural cultivation (NC&G) relative to sites that had never been cultivated (T2&I) (Figure 4-1, Table 4-1). The ChaoI richness

Table 4-1. Estimates of *nifH* diversity made across soil type for sites subject to long-term agricultural cultivation (NC&G) and never cultivated reference sites (T2&I). Comparisons were made at two depths (0-5 cm, and 40-50 cm). OTUs defined by a 93% similarity cutoff.

Treatments	*Total Sequences*	*No. of OTU*	*Chao 1 (mean±s.d.)*	*Chao 1 upper 95%CI*	*Chao 1 lower 95%CI*	*Shannon index H*	*Evenness index J'*
NC&G, 0-5 cm	142	47	112.3±37.1	231.8	70.1	2.98	0.77
NC&G, 40-50 cm	134	33	40.9±6.3	62.5	35.0	3.03	0.87
T2&I, 0-5 cm	134	29	37.3±6.4	60.4	31.2	2.58	0.27
T2&I, 40-50 cm	136	25	29.5±4.8	49.9	25.8	2.84	0.88

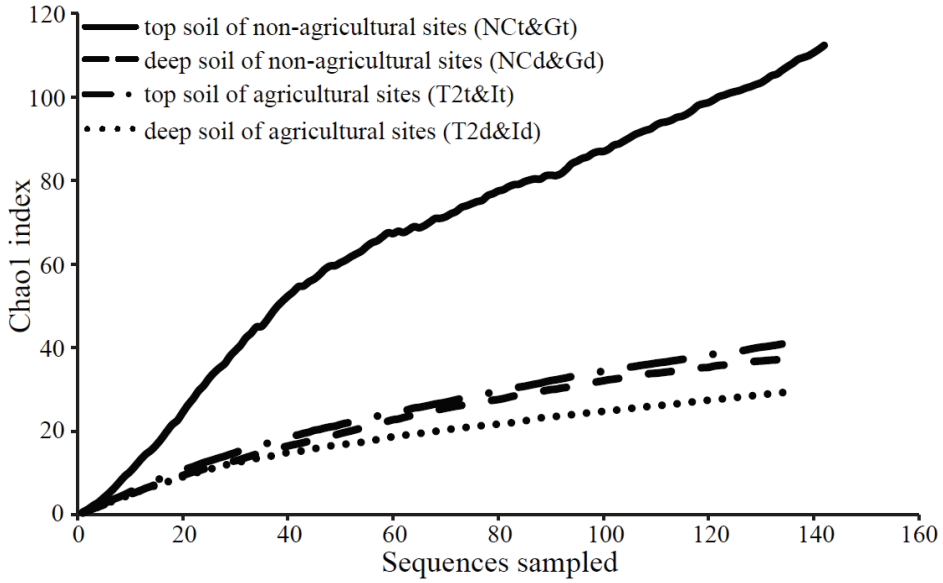

Figure 4-1. Chao I richness estimates for *nifH* clone libraries for 0-5 cm and 40-50 cm soil of agricultural and non agricultural sites with OTUs defined by a 93% DNA similarity cutoff. Confidence intervals are provided in Table 2. OTUs, operational taxonomic units.

estimator indicated that the non-cultivated sites were different from the agricultural sites and this result was significant at 0-5 cm (cultivated: 37.3 ± 6, 31.2 lower confidence interval (LCI), 60.4 higher confidence interval (HCI); never cultivated: 112.3±37, 70.1 LCI, 231,8 HCI) but not at 40-50 cm (cultivated: 29.5±4.8, 25.8 LCI, 49.9 HCI; never cultivated: 40.9±6.3 35.0 LCI, 62.5 HCI). This difference in richness was also observed within each soil type, though the number of sequences examined was not sufficient to establish statistical significance (Table 4-2). ChaoI accumulation curves indicated that the diazotroph communities at 0-5 cm from NC and G were each under sampled and that ChaoI provides only a minimal estimate of richness for each of these samples (Figure 4-2).

Table 4-2 Estimates of *nifH* richness for all soil samples. OTUs defined by a 93% similarity cutoff.

	NCt	*NCd*	*T2t*	*T2d*	*Gt*	*Gd*	*It*	*Id*
Total sequences	74	65	65	66	68	69	69	70
Observed OUT	35	17	17	15	17	22	13	13
Chao 1 (mean±s.d.)	63.6±18	33.0±16	21.2±4	15.5±1	101.5±98	21.1±5	17.2±5	14.2±2
Chao 1 upper 95% CI	121.7	101.8	39.3	23.3	527.9	45.4	38.8	23.4
Chao 1 lower 95% CI	44.4	20.0	17.8	15.3	31.0	23.1	13.7	13.1
Shannon Index H'	3.22	2.29	2.40	2.51	1.5	2.65	1.95	1.99
Evenness J'	0.90	0.81	0.85	0.93	0.53	0.86	0.76	0.78

The impact of depth on the diversity of the diazotrophic community

When examined across all sites *nifH* richness was observed to decline with depth and this difference was significant (Table 4-3, Figure 4-3). The effect of depth on *nifH* richness was also significant when assessed within never cultivated sites (NC&G) but not when assessed within cultivated sites (T2&I) (Table 4-1). The effect of depth was also observed within each individual plot, though depth of sampling was not sufficient to determine statistical significance at this level (Table 4-2). The

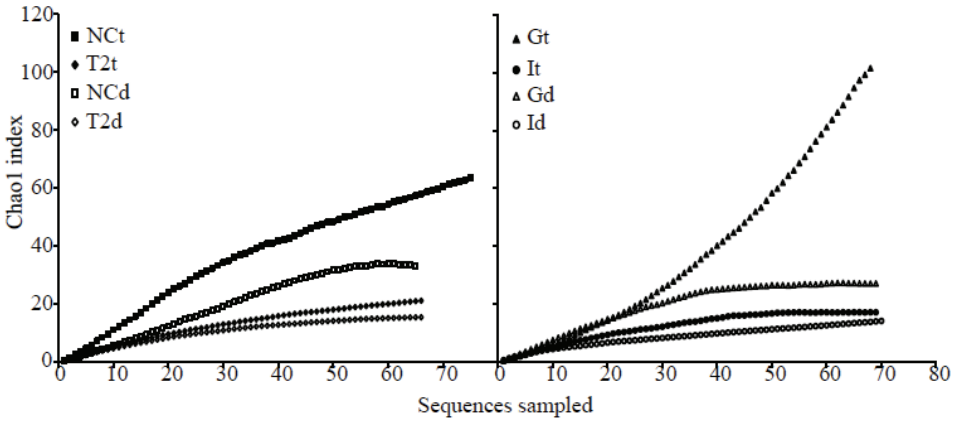

Figure 4-2. Chao I richness estimates for *nifH* clone libraries with OTUs defined by a 93% DNA similarity cutoff. Confidence intervals are provided in Table 1. OTUs, operational taxonomic units.

Table 4-3. Estimates of diversity indexes of *nifH* clone libraries for comparing 0-5 cm and 40-50 cm soil samples from all soil samples. OTUs defined bya 93% similarity cutoff.

Treatments	*Total Sequences*	*No. of OTU*	*Chao 1 (mean±s.d.)*	*Chao 1 upper 95%CI*	*Chao 1 lower 95%CI*	*Shannon index H*	*Evenness index J'*
0-5 cm	276	68	136.1±33.4	236.9	95.4	3.32	0.79
40-50 cm	270	45	51.9±5.6	72.7	46.7	3.29	0.86

coefficient of variation was also calculated for *nifH* richness in the four 0-5 cm communities (C.V. = 0.68) and the four 40-50 cm communities (C.V. = 0.37) indicating that the surface communities are more variable across sites than those present at depth.

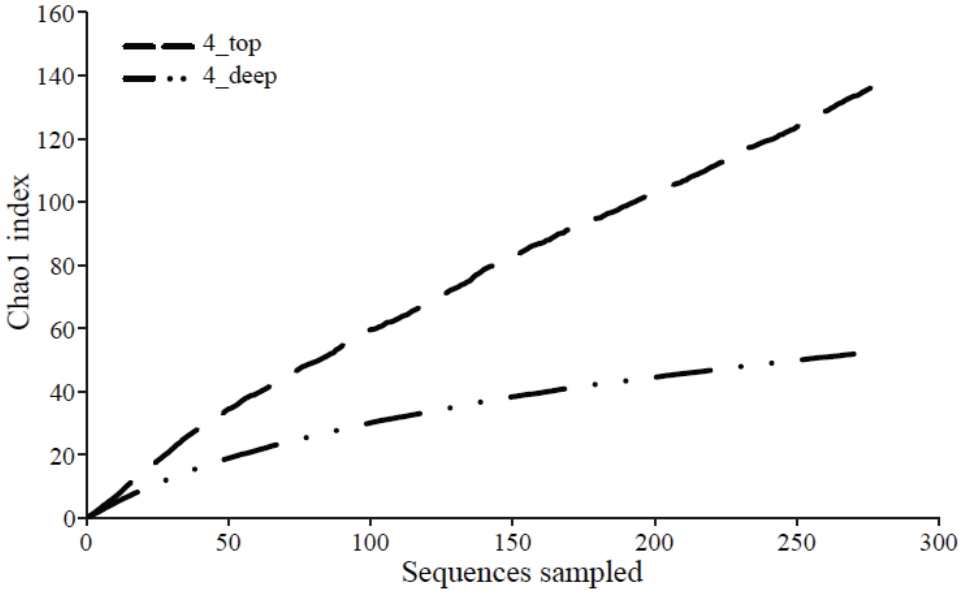

Figure 4-3. Chao I richness estimates compiled with respect to soil depth across all sites examined. OTUs are defined by a 93% DNA similarity cutoff. Confidence intervals are provided in Table 3.

Unifrac analysis of diazotrophic community composition in the sites

Unifrac analysis is sensitive to richness, evenness and composition when information on OTU abundance is considered (weighted analysis) and when OTU abundance is ignored (un-weighted analysis) it is sensitive only to richness and composition. Unifrac provided evidence for the impacts of treatment, depth, and site on diazotroph community composition. Un-weighted analysis revealed that plots within a given soil type clustered together (Figure 4-4A). This result is most likely due to compositional differences between the sites as depth and cultivation history were previously shown to be different with respect to richness. When information on evenness was considered the differences of soil types superseded by treatment and depth effects (Figure 4-4B). The 0-5 cm and 40-50 cm samples from field I clustered together in both un-weighted and weighted analyses. In contrast, at the Chazy site the 40-50 cm samples clustered together in both un-weighted and weighted analyses. The

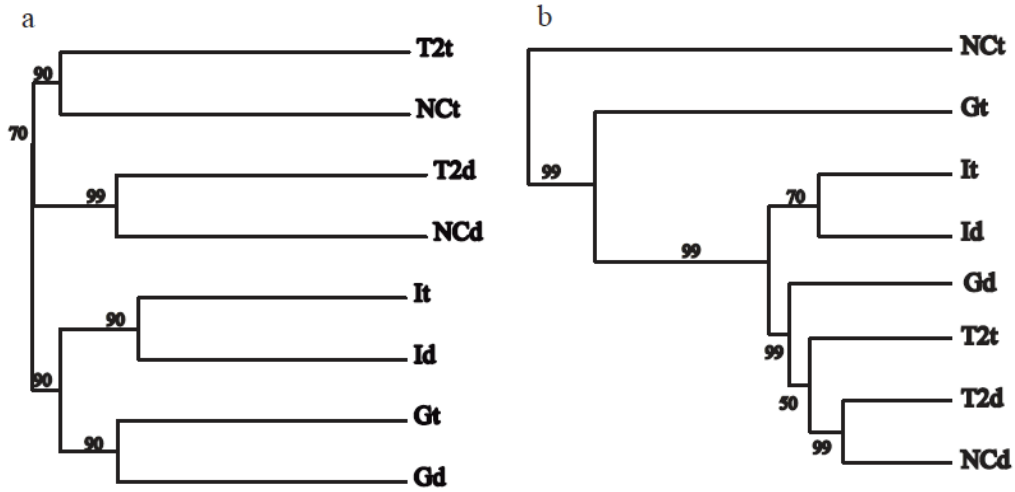

Figure 4-4. Dendograms from UniFrac analyses of *nifH* clone libraries. Numbers indicate the frequency with which nodes were supported by jackknife analysis. Analyses were performed by ignoring OTU abundance (unweighted data, a) and with respect to the abundance of each OTU (weighted data, b)

Sorenson index of similarity, which evaluates the presence or absence of OTUs without respect to abundance, also indicated that the diazotrophic communities in the NC and T2 soils at 40-50 cm (0.45) were more similar than those in the 0-5 cm soils (0.151) consistent with the Unifrac data. The results suggest that communities from deeper soils are more similar to each other than are those form surface soils. The results also show that the communities in the long-term agricultural soils and at 40-50 cm depth share more OTUs than do the surface soils from the never cultivated sites.

The phylogenetic composition of the diazotrophic communities in the sites

The NC 0-5 cm community contained 35 OTUs and 11 of these, representing 49% of the total sequences, belonged to group 1A diazotrophs (Zehr *et al.*, 2003) which is now know to contain sequences from the *Deltaproteobacteria* including *Geobacter* and relatives (Figure 4-5, orange color block). Group 1A sequences were also found in the G 0-5 cm community representing 4 of the 17 OTUs observed and 6% of the sequences recovered. A single 1A sequence was also observed in the G 40-50 cm community, but this group was not observed in other 40-50 cm samples or in any of the agriculturally managed sites. In contrast, there were 13 OTUs that fell into two large clusters (Figure 4-5), both purple color blocks) within the group 1K diazotrophs (which is composed primarily of *Alphaproteobacteria*) which were absent from the NC and G soils at 0-5 cm but were present in all other sites (1K sequences/ total sequences were: 16/65 in T2 0-5 cm, 23/69 in I 0-5 cm, 12/66 in T2 40-50 cm, 38/70 in I 40-50 cm, 31/69 in G 40-50 cm, 5/65in NC 40-50 cm). There were also some OTUs observed only in either 40-50 cm soils (Figure 4-5, blue color blocks), in the Chazy site (Figure 4-5, yellow color blocks), or in the Caldwell site (Figure 4-5), green color blocks). The OTU observed most frequently across all libraries was most closely related to *Rhizobium leguminosarum* bv. *trifolii* (Figure 4-5, Grey color block)

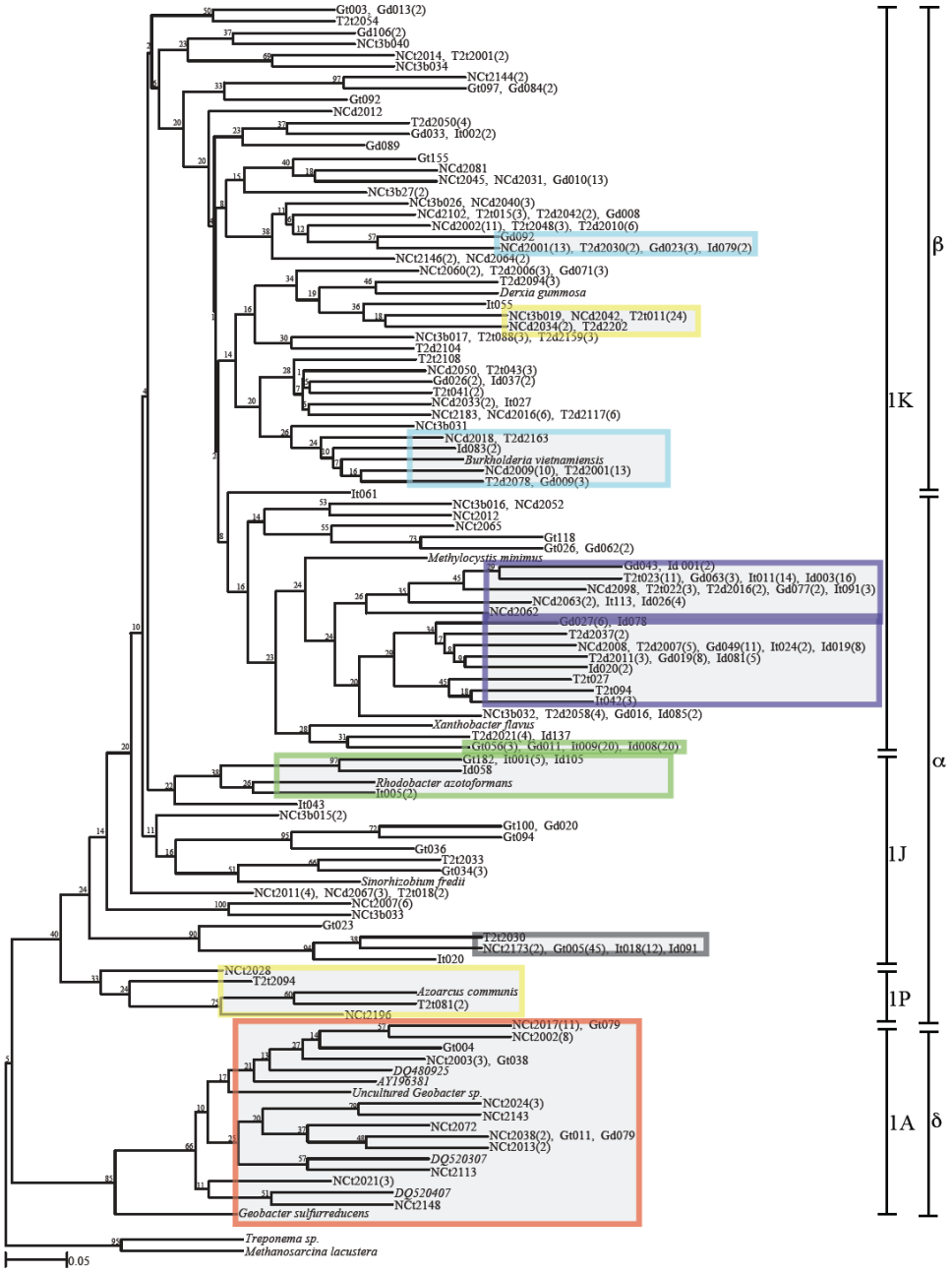

Figure 4-5. Neighbor-Joining tree of *nifH* sequences described in this study. The tree was constructed from 109 amino-acid positions. Each unique OTU is represented in the tree. Branch labels contain multiple sequence names to indicate when an OTU occurs in more than one site. The number in parenthesis indicates the number of sequences observed for each OTU in each site. The names of reference sequences in this study are in italic. The shaded box encompasses the dominant group of OTUs as discussed in the text.

and was observed at 0-5 cm in sites G (45 sequences), I (12 sequences), NC (2 sequences), T2 (1 sequences), and at 40-50 cm in site I (1 sequence). *Rhizobium leguminosarum* bv. *trifolii* is generally found associated with clover and alfalfa. Alfalfa was grown at the Caldwell site several years prior to sampling and clover is widespread at both sites.

Discussion

We found the largest contrast in diazotroph community composition between those at the surface of non-cultivated fields (NC&G 0-5 cm) and those in cultivated fields (T2&I) or in deeper soil layers. These effects were observed in community richness, in OTU composition, in the phylogenetic composition of the communities, and also to a lesser degree in the evenness of these communities. This finding is consistent with previous observations that agricultural management practices reduce the diversity of soil microbial community, including the certain microbial groups which related to the soil nitrogen cycle (Hallin *et al.*, 2009; Shaffer *et al.*, 2000). Previous research has suggested that agricultural management practices (Clegg *et al.*, 2003; Wu *et al.*, 2008), plant varieties (Garbeva *et al.*, 2006; Marschner *et al.*, 2004; Piceno and Lovell, 2000), or both (Kennedy *et al.*, 2004; Kuske *et al.*, 2002) can impact the structure of the microbial community in soils. From our previous results (Chapter 2), we have demonstrated that both tillage and biomass management practices have interacting effects on diazotroph community composition in soil at the Chazy site. Both NC and T2 have the same soil type and have been maintained for more than 30 years without tillage and with plant biomass retained on the soil surface. The major contrast between NC and T2 is that weed management and fertilization have been used to maintain maize as the only plant grown in T2, while the plant

community in NC was not managed and was composed by a diverse mix of grasses and perennial herbaceous plants. Thus, plant effects, fertilization effects, or herbicide effects could all be involved to explain the difference in diazotroph community structure between NC and T2. In NC, however, plant roots rarely extended below 10 cm depth, and the diazotrophic community in the rooting zone differed dramatically from that present below the rooting zone. Similar results were observed for field G as communities in the surface rooting zone were dramatically different from those below the rooting zone and the surface community in soil was dramatically different from the surface community in the adjacent maize field (I). The Caldwell filde G is mantainted as similar as NC which is maintained as a fallow field. Caldwell field I was also treated with fertilizer and herbicide during the maize seasons. Therefore, these results strongly suggest that plant community type was the primary driver of diazotroph diversity.

It is not clear to what degree the impact of plant community on diazotroph diversity was mediated by direct factors such as plant community diversity or the total volume of rhizosphere soil, or by indirect factors related to the impact of the plant community on soil characteristics such as the quantity or quality of soil organic matter. Plant community diversity has been hypothesized to promote microbial diversity by providing a greater diversity of root and litter inputs available to support microbial growth (DeAngelis *et al.*, 2008; Dilly *et al.*, 2004). In addition, high inputs of low diversity organic matter have been observed to reduce the diversity of soil communities (Clegg *et al.*, 2003). Soils at 40-50 cm depth in both the Chazy and Caldwell sites belong to the B horizon in both sites, which have lower organic matter content then the A horizon soils. Differences in organic matter quality can also be observed with depth as labile organic carbon is generally more abundant in the A horizon than in B horizons. These differences are largely driven by organic matter

inputs from plants and have been shown to impact the diversity of the microbial community (Hooper *et al.*, 2000).

The diazotroph community at the surface of Caldwell field G had high richness but was dominated by a single OTU and this dramatically lowered the evenness of this community contrasting with the high evenness observed in the NC surface soils. This OTU was observed to have more than 93% sequence similarity to *R. leguminosarum* bv. trifolii. Both NC and G were observed to contain clover and a member of the *R. leguminosarum* bv. *trifolii* OTU was also observed in the NC site. The fact that only one sequence type was observed for the trifolii-like OTU from field G strongly suggests that a clover root nodule (which could pass through 2 mm mesh) found it was into the DNA extraction made from field G surface soils.

While plant impacts associated with cultivation history and soil depth had the strongest impacts on the diazotrophic community there was also evidence for the effect of soil type/geographic location. Un-weighted Unifrac analysis clustered communities by site regardless of management or depth (Figure 4-4A) and this result was also supported with comparisons between communities made with the Sorenson similarity index. These results suggests that while cultivation history and depth had significant impacts on community richness and on the phylogenetic composition of communities the soil type/geographic location had an impact on the number of OTUs shared between communities, that is sites that were closer together were more likely to share OTUs than sites that were further apart.

In previous research (Hsu and Buckley, 2009), we demonstrated that a single group diazotrophs belonging to the 1K cluster of (containing *Alphaproteobacteria*) was abundant in all maize fields surveyed and was significantly enriched in fields where maize stover was retained. The same OTU was also observed to be abundant in Caldwell field I where maize was grown (26-31% of sequences), and a handful of

sequences from this group were also found in the 40-50 cm samples from NC and G but no sequences were recovered from the surface soils at these sites. In contrast, cluster 1A diazotrophs were uniquely associated with surface soils in NC and G. The only sequences in this group that belong to cultivated isolates fall within the *Deltaproteobacteria* and are most closely related to *Geobacter* spp., however 99% of the 1A *nifH* DNA sequences in GenBank were from uncultivated organisms. In Chapter 2 (Hsu and Buckley, 2009) we show that these group 1A sequences are largely absent from the agricultural fields at the Chazy site. Therefore, we can conclude that 1A diazotrophs were largely absent from maize fields and are likely to be specifically associated with surface rhizosphere soils in grassland sites.

This research demonstrated that long term agricultural sites represent a relatively simple and well defined ecosystem for studying factors that drive the structure of the diazotrophic community. Our results demonstrate that agricultural management practices significantly impact the structure of the diazotrophic community. To further understand the relationship between diazotrophic community composition and its impact on biological nitrogen fixation, it will be necessary to make additional analyses of soil nitrogen fixation and diazotrophic community structure at these sites over time. It would also be useful to develop quantitative PCR assays to evaluate the abundance of specific OTUs in these sites in relation to soil characteristics and N-fixation rates.

REFERENCES

Allison VJ, Miller RM, Jastrow JD, Matamala R, Zak DR (2005). Changes in Soil Microbial Community Structure in a Tallgrass Prairie Chronosequence. *Soil Sci Soc Am J* **69:** 1412-1421.

Buckley DH, Huangyutitham V, Hsu S-F, Nelson TA (2007). Stable Isotope Probing with 15N2 Reveals Novel Noncultivated Diazotrophs in Soil. *Appl. Environ. Microbiol.* **73:** 3196-3204.

Buckley DH, Schmidt TM (2003). Diversity and dynamics of microbial communities in soils from agro-ecosystems. *Environmental Microbiology* **5:** 441-452.

Burgmann H, Widmer F, Von Sigler W, Zeyer J (2004). New molecular screening tools for analysis of free-living diazotrophs in soil. *Appl. Environ. Microbiol.* **70:** 240-247.

Clegg CD, Lovell RDL, Hobbs PJ (2003). The impact of grassland management regime on the community structure of selected bacterial groups in soils. *FEMS Microbiology Ecology* **43:** 263-270.

DeAngelis KM, Brodie EL, DeSantis TZ, Andersen GL, Lindow SE, Firestone MK (2008). Selective progressive response of soil microbial community to wild oat roots. *ISME J* **3:** 168-178.

Dilly O, Bloem J, Vos A, Munch JC (2004). Bacterial diversity in agricultural soils during litter decomposition. *Appl. Environ. Microbiol.* **70:** 468-474.

Drijber RA, Doran JW, Parkhurst AM, Lyon DJ (2000). Changes in soil microbial community structure with tillage under long-term wheat-fallow management. *Soil Biology and Biochemistry* **32:** 1419-1430.

Entry JA, Mills D, Mathee K, Jayachandran K, Sojka RE, Narasimhan G (2008). Influence of irrigated agriculture on soil microbial diversity. *Applied Soil Ecology* **40:** 146-154.

Felsenstein J (2005). PHYLIP (Phylogeny Inference Package), 3.67 ed. Department of Genome Sciences, University of Washington, Seattle.

Finn RD, Mistry J, Schuster-Bockler B, Griffiths-Jones S, Hollich V, Lassmann T (2006). Pfam: clans, web tools and services. *Nucleic Acids Res* **34:** D247-D251.

Garbeva P, Postma J, Veen JAv, Elsas JDv (2006). Effect of above-ground plant species on soil microbial community structure and its impact on suppression of *Rhizoctonia solani* AG3. *Environmental Microbiology* **8:** 233-246.

Girvan MS, Bullimore J, Pretty JN, Osborn AM, Ball AS (2003). Soil Type Is the Primary Determinant of the Composition of the Total and Active Bacterial Communities in Arable Soils. *Appl. Environ. Microbiol.* **69:** 1800-1809.

Hallin S, Jones CM, Schloter M, Philippot L (2009). Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. *ISME J* **3:** 597-605.

Hooper DU, Bignell DE, Brown VK, Brussaard L, Dangerfield JM, Wall DH *et al* (2000). Interactions between Aboveground and Belowground Biodiversity in Terrestrial Ecosystems: Patterns, Mechanisms, and Feedbacks. *BioScience* **50:** 1049-1061.

Hsu S-F, Buckley DH (2009). Evidence for the functional significance of diazotroph community structure in soil. *ISME J* **3:** 124-136.

Hughes JB, Hellmann JJ, Ricketts TH, Bohannan BJM (2001). Counting the Uncountable: Statistical Approaches to Estimating Microbial Diversity. *Appl. Environ. Microbiol.* **67:** 4399-4406.

Karlen DL, Wollenhaupt NC, Erbach DC, Berry EC, Swan JB, Eash NS *et al* (1994). Crop residue effects on soil quality following 10-years of no-till corn. *Soil and Tillage Research* **31:** 149-167.

Kennedy N, Brodie E, Connolly J, Clipson N (2004). Impact of lime, nitrogen and plant species on bacterial community structure in grassland microcosms. *Environmental Microbiology* **6:** 1070-1080.

Konstantinidis KT, Tiedje JM (2005). Towards a Genome-Based Taxonomy for Prokaryotes. *J. Bacteriol.* **187:** 6258-6264.

Kuske CR, Ticknor LO, Miller ME, Dunbar JM, Davis JA, Barns SM *et al* (2002). Comparison of soil bacterial communities in rhizospheres of three plant species and the interspaces in an arid grassland. *Appl. Environ. Microbiol.* **68:** 1854-1863.

Magurran AE (1988). *Ecological diversity and its measurement*. Princeton University Press: Princeton, NJ, 81-101pp.

Marschner P, Crowley D, Yang CH (2004). Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. *Plant and Soil* **261:** 199-208.

Mårtensson L, Díez B, Wartiainen I, Zheng W, El-Shehawy R, Rasmussen U (2009). Diazotrophic diversity, nifH gene expression and nitrogenase activity in a rice paddy field in Fujian, China. *Plant and Soil* **325:** 207-218.

Moebius-Clune BN, van Es HM, Idowu OJ, Schindelbeck RR, Moebius-Clune DJ, Wolfe DW *et al* (2008). Long-term effects of harvesting maize stover and tillage on soil quality. *Soil Sci Soc Am J* **72:** 960-969.

Olsen G, Woese C, Overbeek R (1994). The winds of (evolutionary) change: breathing new life into microbiology. *Journal of Bacteriology* **176:** 1 - 6.

Piceno YM, Lovell CR (2000). Stability in natural bacterial communities: II. Plant resource allocation effects on rhizosphere diazotroph assemblage composition. *Microbial Ecology* **39:** 41-48.

Reed HE, Martiny JBH (2007). Testing the functional significance of microbial composition in natural communities. *FEMS Microbiology Ecology* **62:** 161-170.

Roesch L, Camargo F, Bento F, Triplett E (2008). Biodiversity of diazotrophic bacteria within the soil, root and stem of field-grown maize. *Plant and Soil* **302:** 91-104.

Roesch L, Olivares F, Pereira Passaglia L, Selbach P, de Sá E, de Camargo F (2006). Characterization of diazotrophic bacteria associated with maize: effect of plant genotype, ontogeny and nitrogen-supply. *World Journal of Microbiology and Biotechnology* **22:** 967-974.

Schloss PD (2008). Evaluating different approaches that test whether microbial communities have the same structure. *ISME J* **2:** 265-275.

Schloss PD, Handelsman J (2005). Introducing DOTUR, a Computer program for defining operational taxonomic units and estimating species richness. *Appl. Environ. Microbiol.* **71:** 1501-1506.

Shaffer BT, Widmer F, Porteous LA, Seidler RJ (2000). Temporal and Spatial Distribution of the nifH Gene of N2 Fixing Bacteria in Forests and Clearcuts in Western Oregon. *Microbial Ecology* **39:** 12-21.

Steward GF, Zehr JP, Jellison R, Montoya JP, Hollibaugh JT (2004). Vertical distribution of nitrogen-fixing phylotypes in a meromictic, hypersaline lake. *Microbial Ecology* **47:** 30-40.

Strunk O, and W. Ludwig. (1997). ARB: a software environment for sequence data, 2.5 ed. Department of Microbiology, Technical University of Munich, Munich, Germany.

Swofford DL. (1991). International Society for Microbial Ecology.

Wu L, Ma K, Lu Y (2009). Prevalence of betaproteobacterial sequences in *nifH* gene pools associated with roots of modern rice cultivars. *Microbial Ecology* **57:** 58-68.

Wu T, Chellemi DO, Graham JH, Martin KJ, Rosskopf EN (2008). Comparison of soil bacterial communities under diverse agricultural land management and crop production practices. *Microb. Ecol.* **55:** 293-310.

Zehr JP, Jenkins BD, Short SM, Steward GF (2003). Nitrogenase gene diversity and microbial community structure: a cross-system comparison. *Environmental Microbiology* **5:** 539-554.

CHAPTER 5

EXPLORING RELATIONSHIPS BETWEEN TAXONOMIC DIVERSITY AND THE POTENTIAL NICHE BREADTH OF THE DIAZOTROPH COMMUNITY

Introduction

Many environmental processes are regulated by microorganisms, but interrelations between processes, environmental characteristics, and community composition can be difficult to disentangle. As an added complication, these factors can display tremendous spatial and temporal variation in soils. It can also be difficult to get reliable information on the abundance and activity of specific microorganisms as they occur in their natural habitats. PCR amplification of DNA or RNA sequences is used frequently to study the diversity and abundance of microorganisms in situ. The *nifH* gene, which encodes the reductase subunit of nitrogenase, is commonly used in PCR dependent approaches to study diazotrophs in situ. A number of studies have examined the diversity of key functional genes, such as *nifH*, in relation to environmental processes to determine whether microbial diversity impacts ecosystem function. Evidence for the functional significance of microbial diversity has been obtained for nitrogen fixation (Hsu and Buckley, 2009; Yeager *et al.*, 2004), nitrification (Gieseke *et al.*, 2001; Mertens *et al.*, 2009; Nugroho *et al.*, 2009), denitrification (Enwall *et al.*, 2005; Rich *et al.*, 2003; Song *et al.*), and for the anaerobic oxidation of ammonia (Kuypers *et al.*, 2003; Schubert *et al.*, 2006). While these studies revealed associations between community diversity and ecological activity they did not attempt to reveal the mechanisms which underlie these associations. In fact, the mechanisms which underlie relationships between community structure and activity remain poorly characterized. There are many ways in which increasing diversity may impact ecological processes including: functional redundancy, niche breadth, synergistic associations, and idiosyncratic compositional

effects. These types of questions are difficult to evaluate with cultivation independent techniques applied *in situ*.

From our previous results, we demonstrated that diazotroph community structure was influenced by agricultural management and that variation in diazotroph community structure impacts soil N-fixation rates. Here we performed a series of laboratory experiments to explore the mechanisms by which N-fixation rate is influenced by diazotroph diversity. Experiments include cultivation and microcosm experiments designed to evaluate niche occupancy and functional resistance as a function of community diversity. In particular we test the hypotheses that: 1) greater diversity in the diazotrophic community will lead to greater capacity to respond to different growth conditions; and 2) resistance of N-fixation to perturbation will increase with the diversity of the diazotroph community.

Material and methods

Most probable number experiments

Soil samples were taken from Chazy treatments T2, T4, and NC on 11/02/2008 for Experiment A and on 10/07/2009 for Experiment B. These treatments have diazotroph community diversity that ranges from high richness and high evenness (NC: Chao1 = 64 ± 18, H' = 3.22, J' = 0.9), to moderate richness and evenness (T2: Chao1 = 21 ± 4, H' = 2.4, J' = 0.85), and low richness and low evenness (NC: Chao1 = 8 ± 1, H' = 1.14, J' = 0.53) (Hsu and Buckley, 2009). A total of 15 soil cores of 0-5 cm depth were taken from each replicate plot and these samples were sieved through 2 mm sieve and homogenized. Soils were stored at ambient temperature and experiments were initiated as described below within 2 days of sampling.

An experiment was designed using the most probable number (MPN) technique to evaluate the range of conditions that support the growth of N-fixing bacteria in soils that have different diversities of diazotrophs. The soil samples were diluted and used to inoculate a modular nitrogen free media (described below) in which carbon source, pH, and trace element composition was varied. One gram of homogenized soil from each replicate plot was serially diluted in buffer (1L containing: 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \bullet 7H_2O$, 0.1 g NaCl, 0.02 g $CaCl_2 \bullet 2\ H_2O$) to provide inoculum density of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} g soil. Experiments were performed in 96 well plates (Costar, Corning) and all dilutions for each condition were replicated three times. Plates were then incubated at different levels of temperature and oxygen (as described below). Optical density was monitored at 590 nm for 45 days using a 96 well plate reader. The threshold of detection was determined to be 5 x 10^4 cells g^{-1} and values below this threshold were treated as non-detect.

The modular medium was a nitrogen-free (n-free) mineral medium modified from Noar and Buckley (Noar and Buckley, 2009) which contained per L: 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \bullet 7H_2O$, 0.1 g NaCl, 0.02 g $CaCl_2 \bullet 2\ H_2O$, 2 ml minor element solution (containing per L: 0.8 mg $CuSO_4 \bullet 5\ H_2O$, 22mg $ZnSO_4 \bullet\ 7\ H_2O$, 0.36mg $Na_2B_4O_7$, 3.1 mg $ZnSO_4 \bullet 7\ H_2O$, 10mg $FeSO_4 \bullet 7\ H_2O$, 5 mg EDTA), and 1 ml vitamin solution (containing per L: 100 mg biotin and 200 mg pyridoxol HCl). In order to vary trace element composition the media was prepared either as above (Fe only) or with the addition of 1 ml of 10 mM NaO_2V (Fe + V), or with addition of both 1 ml of 10 mM NaO_2V and 10 mM $Na_2MoO_4 \bullet\ 2H_2O$ (Fe + V + Mo). Eight different carbon sources were used to support growth including: malic acid (C1), cellobiose (C2), sucrose (C3), mannitol (C4), vanillin (C5), sodium acetate (C6), xylose (C7), and glycerol (C8). Carbon sources were supplied at 37 mM. When needed the pH of the media was adjusted by including either 100 mM MOPS pH 7.5, 100 mM MES pH 5.5,

or by titrating to pH 3.5 with HCl. Anoxic incubation was performed using GasPak™ EZ Anaerobe Container System Sachets and Anaerobic chambers (BD, Franklin Lakes, NJ); low oxygen incubation was performed using the BD GasPak™ EZ CampyPouch™ System (which achieves 5% O_2).

MPN experiment A included six different carbon sources (C1- C6), 4 different temperatures (4ºC, 22ºC, 30ºC and 41ºC), 3 pH levels (pH 3.5, pH5.5 and pH7.5), three different trace element conditions, and three levels of oxygen (atmosphere air, low oxygen, and anoxic). The experiment was not fully factorial in design and a total of 60 different growth conditions were tested in MPN experiment A (Table 5-1). The design of MPN experiment B was varied and included five different carbon sources (C2, C3, C4, C7 and C8), with two pH levels (pH 7.5 and pH 5.5), and crossing 3

Table 5-1. Design and results from MPN experiment 1. Values indicate the number of replicate plots (n = 4) that were positive for growth in N-free media. Each value indicates growth in a particular set of conditions (inocula, carbon source, and incubation condition) as defined by the position in the grid. The bottom row indicates the number of conditions that supported growth across the 4 replicate plots of each field treatment.

Treatment	*T4 (nTB)*						*T2 (nTnB)*						*NC*					
Carbon source	*C1*	*C2*	*C3*	*C4*	*C5*	*C6*	*C1*	*C2*	*C3*	*C4*	*C5*	*C6*	*C1*	*C2*	*C3*	*C4*	*C5*	*C6*
Incubation condition																		
pH 7.5 22ºC	0	0	3	1	0	0	0	1	4	2	0	0	0	4	1	4	0	4
pH 7.5 4ºC	3	1	4	1	0	3	2	0	1	1	0	1	0	0	4	0	0	0
pH 7.5 30ºC	0	0	4	4	0	0	0	4	2	3	0	4	0	4	4	4	0	4
pH 7.5 41ºC	1	3	4	2	0	2	0	0	4	2	0	3	0	2	0	0	0	0
pH 5.5 22ºC	0	0	0	3	0	1	0	0	3	4	0	1	1	3	1	4	0	2
pH 3.5 22ºC	0	0	1	3	0	3	0	0	1	3	0	1	1	3	4	4	0	4
pH 7.5 22ºC no Mo	0	4	3	2	0	4	0	0	3	0	0	4	0	3	4	2	0	4
pH 7.5 22ºC no Mo, noV	1	0	4	4	0	3	3	0	2	4	0	0	2	1	4	4	0	4
pH 7.5 22ºC low oxygen	1	4	4	4	0	0	2	4	4	4	0	0	1	4	4	4	0	1
pH 7.5 22ºC anoxic	0	0	0	0	0	0	2	0	1	0	0	0	0	2	2	3	0	0
Total	85						80						111					

oxygen levels (atmosphere air, low oxygen levels and anoxic). Thus a total of 30 growth conditions were tested in MPN experiment B (Table 5-2). The Tukey test was

used to contrast the number of conditions observed to support the growth of putative diazotrophs.

Table 5-2. Design and results from MPN experiment 2. Values indicate the number of replicate plots (n = 4) that were positive for growth in N-free media. Each value indicates growth in a particular set of conditions (inocula, carbon source, and incubation condition) as defined by the position in the grid. The bottom row indicates the number of conditions that supported growth across the 4 replicate plots of each field treatment.

Treatment	*T4 (nTB)*					*T2 (nTnB)*					*NC*				
Carbon source	*C2*	*C3*	*C4*	*C7*	*C8*	*C2*	*C3*	*C4*	*C7*	*C8*	*C2*	*C3*	*C4*	*C7*	*C8*
Incubation condition															
pH 7.5, 22ºC, oxygen	4	2	3	1	1	4	2	4	0	2	4	1	0	1	1
pH 7.5, 22ºC, low oxygen	1	2	2	1	3	3	0	1	0	0	4	4	4	3	4
pH 7.5, 22ºC, anoxic	2	0	0	0	0	0	0	0	0	0	1	0	0	0	0
pH 5.5, 22ºC, oxygen	1	0	0	0	1	2	0	0	0	0	1	3	2	0	0
pH 5.5, 22ºC, low oxygen	4	3	4	3	2	1	1	0	0	0	4	4	4	4	4
pH 5.5, 22ºC, anoxic	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	43					20					53				

Microcosm experiments

Microcosm experiments were performed in parallel with the MPN experiments described above. Five gram of soil from each replicate of each treatment was placed into 18 × 150 mm Balch tubes (Bellco Glass, Vineland, New Jersey), and the headspace was replaced with synthetic air containing 20% O_2 and 80% $^{15}N_2$ (>98 atom % ^{15}N, Isotec, Sigma-Aldrich). Controls were processed in parallel and received unlabeled N_2 gas. Unless otherwise note soils were incubated at 21°C. Six sets of conditions were used for microcosm experiment A: no addition control (no add); elevated temperature (41°C), with soils incubated at 41ºC; freeze thaw (F/T), with soils subjected to three cycles of freezing at -80°C for 1 hour and thawing at 37°C for 15 min; anoxic incubation (anoxic); increased moisture (+H_2O), with soils wetted by addition of 1ml of sterile water; and low pH (pH 4.2), with soil pH adjusted to 4.2 with acetic acid. Microcosm experiment B included four sets of conditions with two levels of substrate: no addition control (no add), or carbon source mixture added

(Cmix); and two levels of oxygen: air or anoxic. The added carbon sourse mixture contained: 5% (w/w) of C3, C4, C7, C8 and 1% (w/w) of C2.

All microcosm tubes were incubated horizontally at room temperature in the dark for 9 days. The atom % ^{15}N of soil samples was determined using a Finnigan MAT Delta Plus mass spectrometer (Thermo Electron Corporation, Waltham, Massachusetts) plumbed to a Carlo Erba NC2500 elemental analyzer (CE Instruments, Wigan, UK) through a Conflo II open split interface for elemental and isotopic composition of solid samples (Thermo Electron Corporation). The net potential N-fixation rate was calculated from the difference of total ^{15}N in soils receiving $^{15}N_2$ relative to the parallel controls.

Results and discussion

MPN Experiment 1

The results from Chapter 2 reveal that the diazotrophic community in the never cultivated field site (NC) had greater richness and evenness and was different in composition from the agriculturally managed sites T4 and T2. In addition, the richness of the diazotroph community in T2 was greater than that in T4 though these communities were more similar to each other in composition than either was to the diazotroph community in NC. The hypothesis that we sought to test is that greater diversity in the diazotrophic community will correspond to a greater number of conditions that can support the growth of N-fixing bacteria. The different conditions used in the MPN experiment were used as a proxy to represent potential ecological niches that could exist in soil. The results provided weak support for the hypothesis. The NC soils supported growth of N-fixing organisms under a greater diversity of conditions than was observed in T2 and T4 (Table5-1 and Table 5-3), however no

Table 5-3. Results from MPN experiment 1 expressed for field replicates. Values are the number of conditions found to support growth in N-limited media. Different letters indicate samples that differ significantly ($p < 0.05$).

Treatment	*replicates*	*Growth conditions*	*Average ± s.d.*	*Tukey test*
T4	*r1*	21		
	r2	20		
	r3	20		
	r4	24	21.25±1.892	B
T2	*r1*	17		
	r2	21		
	r3	19		
	r4	23	20.00±2.582	B
NC	*r1*	28		
	r2	30		
	r3	28		
	r4	25	27.75±2.062	A

significant difference was observed in the number of conditions that supported growth in T2 and T4 suggesting that richness was not a key factor in the difference observed. This pattern of results would suggest that composition rather than richness itself dictates the ability of the community to support N-fixation under different growth conditions.

Microcosm Experiment 1

Microcosm experiment 1 was conducted in parallel with MPN experiment 1. If greater diversity in the diazotroph community conveys greater resistance to disturbance we would expect less variation in N-fixation in NC soils than in T2 or T4, and less variation in T2 than in T4. However, greater variation in N-fixation rates was observed in response to perturbation in NC relative to T4 or T2 (Figure 5-1). Therefore, NC, which contained highest diversity of diazotrophic community, did not have higher resistance for N-fixation rates under the conditions tested. As discussed above, NC also had significant compositional difference in diazotrophic community

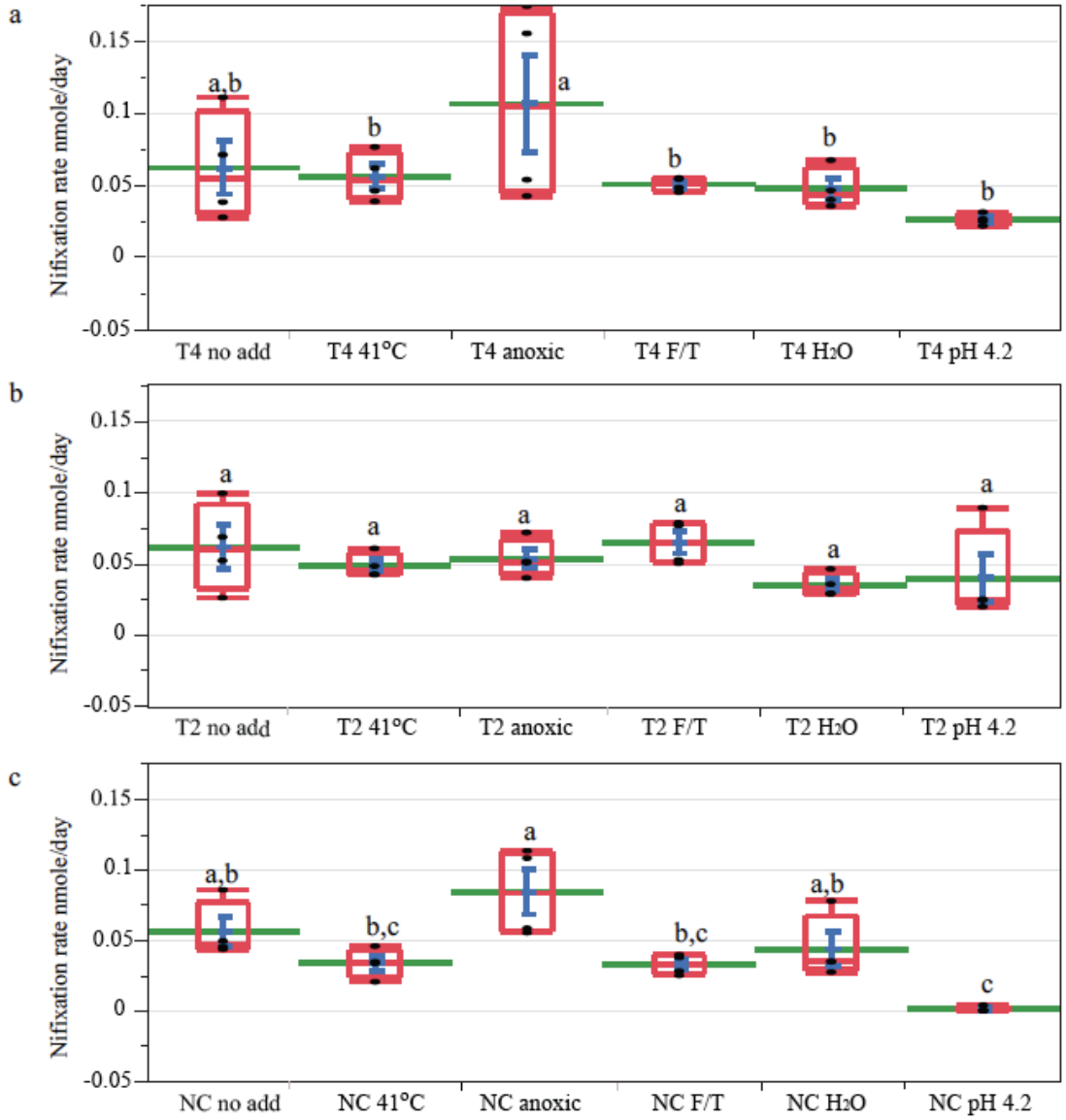

Figure 5-1. N- fixation rates of soil samples from 11/02/2008 under different incubation conditions: a) was soil from T4, b) was from T2 and c) was from NC. Treatments are described in the methods. Red boxes are 95% quantiles: black dots are individual points; green line is the mean, and blue line is the standard deviation; different letters are used to represent differences that are significant as revealed by all pair Turkey test at $p < 0.05$.

structure from T2 and T4. This result suggests that community composition rather than richness may determine the way that the community responds to stimuli.

MPN Experiment 2

We sought to repeat and refine the MPN experiment described above by altering the growth conditions to exclude conditions that did not support growth in any site and adding new C sources expected to support growth of N-fixing organisms. In addition, greater focus was placed on oxygen and pH as main factors in the experiment (Table 5-2) since the earlier experiment suggested that varying these factors had an impact on the growth of diazotrophs. Again, we observed that NC soils supported growth under the greatest diversity of conditions (Table 5-4). No significant difference was observed between T2 and T4 (Table 5-4). These results are consistent with those described above suggesting that composition rather than richness governs the functional breadth of the N-fixing community. The results also suggested that a greater number of N-fixing organisms were able to grow when incubated under low oxygen conditions than when grown in air or in anoxic conditions (Table 5-2 & Table 5-4).

Table 5-4. Results from MPN experiment 2 expressed for field replicates. Values are the number of conditions found to support growth in N-limited media. Different letters indicate samples that differ significantly ($p < 0.05$).

Treatment	*replicates*	*# of growth*	*Avg # of growth condition in each treatment*	*Turkey test*
T4	*r1*	12		
	r2	11		
	r3	10		
	r4	10	10.75 ± 0.957	B
T2	*r1*	3		
	r2	9		
	r3	5		
	r4	3	5.00 ± 2.828	B
NC	*r1*	13		
	r2	13		
	r3	14		
	r4	13	13.25 ± 0.500	A

Microcosm Experiment 2

The carbon source mixture added to the soil microcosms resulted in significant stimulation of N-fixation in all soils and conditions tested (Figure 5-2). In general, the stimulation of N-fixation was highest in NC, next highest in T2, and lowest in T4 (Figure 5-2). In air, the stimulation observed in NC and T2 was greater than that observed in T4 and these results were significant (Table 5-2). In the absence of oxygen, the stimulation in NC was significantly greater than that in T2 and T4 but the difference observed between T2 and T4 was not significant. Results from this set of microcosm tests suggested that higher richness in the diazotrophic community conveyed on the soil a greater capacity to respond when faced with a diverse mixture of carbon substrates. However, due to the differences in the composition of the diazotrophic community between NC and the agricultural sites (T2 and T4), we cannot exclude the possibility that this response was mediated be idiosyncratic differences in community composition.

Conclusion

Torsvik (Vitousek *et al.*, 2002) has reviewed the potential mechanisms through which changes in microbial diversity may influence microbial functions in the environment. Yet, there remains conflicting evidence as to the degree to which microbial diversity impacts ecological processes. Some results suggest that microbial diversity contributes to resilience in soil processes, resistance to perturbation, and may be positively correlated with the rates of certain soil processes (Degens *et al.*, 2001; Girvan *et al.*, 2005; Griffiths *et al.*, 2000). In contrast, other studies have failed to reveal a relationship between microbial diversity and soil functions (Degens, 1998;

Nannipieri *et al.*, 2003; Wertz *et al.*, 2007). The research described in this chapter did not support the hypothesis that the richness of the diazotroph community was

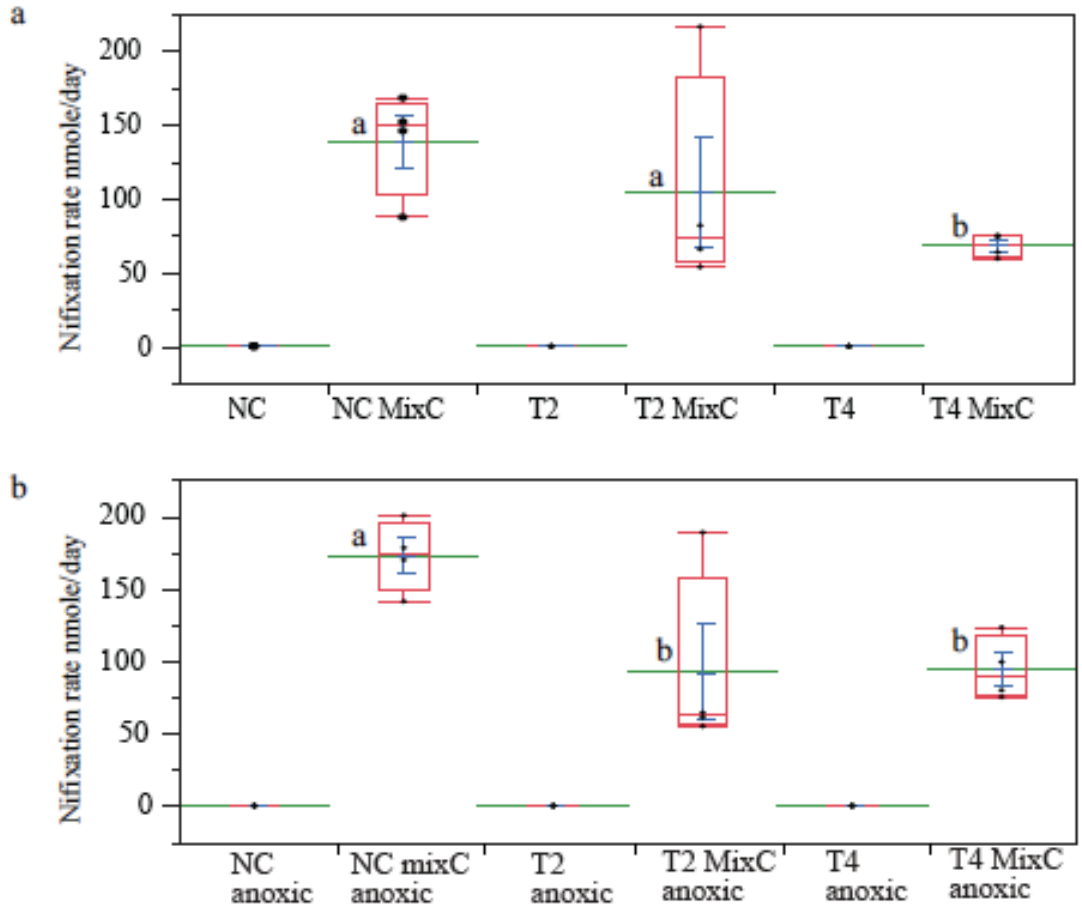

Figure 5-2. N- fixation rates of soil samples from 10/07/2009 under different incubation conditions: a) microcosms incubated under air, b) microcosms incubated under anoxic conditions. MixC indicates samples where the mixed carbon substrate was added. Red boxes are 95% quantiles: black dots are individual points; green line is the mean, and blue line is the standard deviation; different letters are used to represent differences that are significant as revealed by all pair Turkey test at $p < 0.05$.

correlated with a greater functional breadth of the diazotroph community, or that richness provided greater resistance to perturbation. The results, however, were consistent with the conclusion that the functional capacity of the N-fixing community was impacted by the differences in community composition.

REFERENCES

Degens BP (1998). Decreases in microbial functional diversity do not result in corresponding changes in decomposition under different moisture conditions. *Soil Biology and Biochemistry* **30:** 1989-2000.

Degens BP, Schipper LA, Sparling GP, Duncan LC (2001). Is the microbial community in a soil with reduced catabolic diversity less resistant to stress or disturbance? *Soil Biology and Biochemistry* **33:** 1143-1153.

Enwall K, Philippot L, Hallin S (2005). Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization. *Appl. Environ. Microbiol.* **71:** 8335-8343.

Gieseke A, Purkhold U, Wagner M, Amann R, Schramm A (2001). Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm. *Appl. Environ. Microbiol.* **67:** 1351-1362.

Girvan MS, Campbell CD, Killham K, Prosser JI, Glover LA (2005). Bacterial diversity promotes community stability and functional resilience after perturbation. *Environmental Microbiology* **7:** 301-313.

Griffiths BS, Ritz K, Bardgett RD, Cook R, Christensen S, Ekelund F (2000). Ecosystem response of pasture communities to fumigation-induced microbial diversity reductions: an examination of the biodiversity- ecosystem function relationship. *Oikos* **90:** 279-294.

Hsu S-F, Buckley DH (2009). Evidence for the functional significance of diazotroph community structure in soil. *ISME J* **3:** 124-136.

Kuypers MMM, Sliekers AO, Lavik G, Schmid M, Jorgensen BB, Kuenen JG *et al* (2003). Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature* **422:** 608-611.

Mertens J, Broos K, Wakelin SA, Kowalchuk GA, Springael D, Smolders E (2009). Bacteria, not archaea, restore nitrification in a zinc-contaminated soil. *ISME J* **3:** 916-923.

Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G (2003). Microbial diversity and soil functions. *European Journal of Soil Science* **54:** 655-670.

Noar JD, Buckley DH (2009). Ideonella azotifigens sp. nov., an aerobic diazotroph of the betaproteobacteria isolated from

grass rhizosphere soil, and emended description of the genus Ideonella. *International Journal of Systematic and Evolutionary Microbiology* **59:** 1941-1946.

Nugroho RA, Rˆling WFM, van Straalen NM, Verhoef HA (2009). Changes in nitrification and bacterial community structure upon cross-inoculation of Scots pine forest soils with different initial nitrification rates. *Soil Biology and Biochemistry* **41:** 243-250.

Rich JJ, Heichen RS, Bottomley PJ, Cromack K, Jr., Myrold DD (2003). Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. *Appl. Environ. Microbiol.* **69:** 5974-5982.

Schubert CJ, Durisch-Kaiser E, Wehrli B, Thamdrup B, Lam P, Kuypers MMM (2006). Anaerobic ammonium oxidation in a tropical freshwater system (Lake Tanganyika). *Environmental Microbiology* **8:** 1857-1863.

Song K, Lee S-H, Mitsch WJ, Kang H Different responses of denitrification rates and denitrifying bacterial communities to hydrologic pulsing in created wetlands. *Soil Biology and Biochemistry* **In Press, Corrected Proof**.

Vitousek PM, Cassman K, Cleveland C, Crews T, Field CB, Grimm NB *et al* (2002). Towards an ecological understanding of biological nitrogen fixation. *Biogeochemistry* **57-58:** 1-45.

Wertz S, Degrange V, Prosser JI, Poly F, Commeaux C, Guillaumaud N *et al* (2007). Decline of soil microbial diversity does not influence the resistance and resilience of key soil microbial functional groups following a model disturbance. *Environmental Microbiology* **9:** 2211-2219.

Yeager CM, Kornosky JL, Housman DC, Grote EE, Belnap J, Kuske CR (2004). Diazotrophic community structure and function in two successional stages of biological soil crusts from the Colorado Plateau and Chihuahuan Desert. *Appl. Environ. Microbiol.* **70:** 973-983.

CHAPTER 6

CONCLUSIONS

Chapter 2 provided a detailed look at the diazotroph community in the Chazy long term agricultural site in samples from November 2005. Tillage had sweeping impacts on soil characteristics but the main effect of tillage on N-fixation rates was not significant. Instead, significant variation in N-fixation rates was primarily associated with biomass management. The effect of tillage on N-fixation rates was observed to interact with the effect of biomass management. Tillage and biomass management also had interacting effect on diazotrophic community structure. Biomass retention caused a dramatic change in the evenness of the diazotroph community as maize residue retention led to a significant enrichment in the frequency of specific OTUs associated with the *Rhizobiales*. Tillage had an impact on the composition of the community that could be observed only when frequency information was ignored. The never cultivated control site had significantly greater richness than the agricultural sites and dramatically different composition. Diazotrophs related to *Geobacter* and relatives from the *Deltaproteobacteria* represented a large fraction of the community in the control site but were nearly absent in the agricultural fields. The results suggested that differences in diazotroph community structure at the site have functional significance, influencing rates of N-fixation.

Chapter 3 reports the relationship between N-fixation rate, soil characteristics, and diazotrophic community structure in soil over time. *nifH* TRFLP was used to analyze the composition of the soil diazotrophic community under different agricultural treatments and times. An AMMI model was used to partition sources of

variance in the diazotroph community across samples. The greatest amount of variation in the diazotrophic community was explained by a Interaction principle component associated with time of sampling (explaining 24% of the variation in community structure), the next greatest source of variation (explaining 9% of the variation in community structure) was explained by a principle coordinate that varied significantly in relation to tillage, and the third largest source of variation (explaining 7% of the variation in community structure) was explained by a principle coordinate that varied significantly in relation to biomass management. These patterns of variation suggest that the effect of time, tillage, and biomass management all impact the composition of the microbial community but that these factors interact in their impacts on the community.

A general linear regression (GLM) model was used to evaluate the impacts of diazotrophic community, soil characteristics, treatment, and time on N-fixation rates. The GLM model made it possible to partition the effects of time, diazotrophic community, soil characteristics, and agricultural treatments on N-fixation rates. Time accounted for the greatest variation in N-fixation rates followed by diazotrophic community composition, soil characteristics, and treatments. A second GLM model was constructed in which time and treatments were excluded because these factors were previously shown to co-vary with variation in soil characteristics and diazotroph community composition. This second model also confirmed that variation in diazotroph community composition explains a greater proportion of the variance in N-fixation rates than can be explained by variation in soil characteristics alone. This suggests that changes that occur in the diazotroph community over time are major drivers of N-fixation rates. Therefore, the diazotroph community composition was sensitive to treatment effects leading to interactions between treatment and time and

changes in the structure of the diazotrophic community explain significant variation in soil N-fixation rates.

Chapter 4 explored factors that impact diazotrophic community structure at different agricultural management sites. In this chapter we contrast the Chazy site with a separate long-term agricultural management site at Caldwell field in Ithaca, Tompkins, NY. Caldwell field has sites under maize that have been consistently cultivated for more than 35 yrs (though under considerably different tillage and biomass management routines from Chazy) and also contains adjacent fields that have never been cultivated. The chapter reveals that the contrasts observed in diazotroph community composition between cultivated and non-cultivated fields at Chazy are also present at Caldwell. Agricultural management with maize clearly leads to a dramatic reduction in the diversity of the diazotrophic community. While the contrast between cultivated and never cultivated fields was stark within each site, there was also evidence that differences in diazotroph community composition were driven to a degree by geographic location (possibly associated with soil type).

The results suggest the hypothesis that plant characteristics are a major driver of the difference seen in the diazotrophic community between the long term agricultural fields and their never cultivated counterparts. The never cultivated fields were dominated by a diverse mixture of grasses and herbaceous plants while the agricultural fields contained almost exclusively maize. The diversity of the diazotroph community also dropped in samples taken below the rooting zone in the never cultivated fields. It is not clear to what degree the impact of plant community on diazotroph diversity was mediated by direct factors such as plant community diversity or the total volume of rhizosphere soil, or by indirect factors related to the impact of the plant community on soil characteristics such as the quantity or quality of soil

organic matter. In both sites, we found diazotrophic groups that were specific either to maize fields or to the grassy control plots. A distinct group of *nifH* sequences associated with iron reducing *Deltaproteobacteria* was largely unique to samples taken from the rooting zone in the grassy fields. The nature of this association, and whether there is a specific association between this group and perennial grasses, remains to be determined.

Chapter 5 explored the hypothesis that the impact of diazotroph diversity on N-fixation rates is mediated through functional diversity and functional redundancy. Cultivation based experiments were used to determine whether soils with a greater diversity of diazotrophs also support the growth of organisms under a greater variety of conditions when N is limiting. When N was limiting, the diazotrophic community in NC contained organisms able to grow under a greater diversity of conditions than was observed for the communities from T2 or T4. This result could be due either to the richness of the NC community or it may be due to compositional differences between NC and the agricultural sites. The ability to grow under a more diverse set of conditions may translate into the ability to fix N under a greater diversity of environmental conditions (to occupy a greater variety of ecological niches) in NC than in the agricultural sites. Microcosm experiments suggested that the addition of a mixture of carbon sources led to greater stimulation of N-fixation in NC than in the agricultural sites, suggesting that the greater diversity in NC may have allowed this community greater ability to respond to this heterogeneous carbon amendment. Other microcosm experiments failed to demonstrate greater resistance to perturbation in NC relative to the agricultural sites. The results suggest that the same perturbation or incubation conditions applied to different communities of diazotrophs will provoke distinct responses from the diazotrophic community. This finding supports the

hypothesis that the structure of the diazotroph community has a significant impact on N-fixation rates, though it is not clear to what degree differences in richness or idiosyncratic differences in composition drive these impacts.

APPENDIX A

Application of $^{15}N_2$ for measuring potential nitrogen fixation rates in soil

Introduction

The accurate determination of *in situ* N-fixation rates is complicated by many factors as reviewed in Chapter 1. In particular, the acetylene reduction assay (ARA) has proved relatively unreliable in soil systems due to large variations observed in the acetylene/N_2 reduction ratio. It is also possible to use fixation of ^{15}N-labeled dinitrogen into soil as a direct measurement of soil N-fixation. This method is more direct than ARA and was actually developed prior to introduction of the ARA method. A disadvantage of this approach is that it is less sensitive than acetylene reduction and thus incubations must be carried out for longer periods of time than is required for ARA measurements. The need for prolonged (multiple day) incubations in gas tight chambers raises the possibility of bottle effects and thus $15N_2$ fixation rates must be treated as 'potential' N-fixation rates. Such limitations are not unique to this approach as potential rate measurements are used widely in microbial ecology in a variety of applications.

We used $^{15}N_2$ fixation measurements as our primary method for determining potential N-fixation rates in this dissertation. The method is described in Chapter 2, but briefly, soils were incubated in the presence of >98% atom enriched $^{15}N_2$ containing air for 9 days and then the atom fraction ^{15}N of the soil was determined by mass spectrometry. To calculate the amount of $^{15}N_2$ fixed into soil it was necessary to know the background $\delta^{15}N$ enrichment of soils and so controls were incubated in parallel with unlabeled N_2. In this appendix we describe experiments performed to verify that the technique we used was responsive to treatments known to either inhibit or stimulate N-fixation. Further discussion of this approach is provided in Chapter 1

and detailed information on the method of measuring $^{15}N_2$ fixation is provided in Chapter 2.

Results & Discussion

Experiment 1: Chazy Soil

Five grams of sieved soil (from Chazy treatment T2 sampled on 09/25/2006) were placed in each tube. Each set of treatments was represented by 6 tubes: one set of three replicates incubated with a headspace of 20% $^{15}N_2$ and 80% O_2, and another parallel set of three tubes with a headspace of 20% N_2 and 80% O_2. All microcosms were incubated at room temperature under dark for 9 days and other experimental details are as described in Chapter 2. Treatments were as follows:

Control : soil at field moisture with no additions

Autoclaved: Tubes containing 5 g soil were autoclaved for 3 hrs immediately prior to the start of the experiment.

Water only: 500 μl of sterile water was added to 5 g soil

Glucose: 500 μl of glucose (25% w/v) was added into 5 g of soil (25 mg glucose g^{-1} soil final).

Nitrogen: 500 μl of NH_4NO_3 (3 mg/ml) added into 5g of soil (300 μg NH_4NO_3 g^{-1} soil final).

The addition of water to dry soil was observed to result in stimulation of N-fixation and this result was significant (Table 1). Autoclaving dry soil was observed to reduce N-fixation significantly (Table 1) but did not eliminate N-fixation. Relative to water only controls, the addition of ammonium nitrate reduced N-fixation and the addition of glucose increased N-fixation and these results were significant (Table 1).

Table 1. N-fixation rates from Chazy soil, Experiment 1

Treatment	*N-fixed* ($\mu g\ kg^{-1}\ d^{1}$)
Control	350 ± 4.5[2]
Autoclaved	133 ± 10[1, 2]
Water only	399 ± 50[1]
Glucose	4867 ± 903[1, 2]
Nitrogen	337 ± 12[1, 2]

[1] Values different from 'Control' by U-Test at $P < 0.05$.
[2] Values different from 'Water only' by U-Test at $P < 0.05$.

Experiment 1 revealed that N-fixation, as measured by $^{15}N_2$ incorporation into soil, responded as expected being inhibited by autoclaving and addition of excess nitrogen and stimulated by the addition of labile carbon. Some concerns remained however, as neither autoclaving nor addition of N resulted in complete inhibition of N-fixation. It is well known that dry soils are difficult to sterilize by autoclave and from the literature it can be predicted that 3 hrs of autoclaving would be insufficient to sterilize dry soil. Autoclaving would also be expected to increase the availability of labile carbon released from lysed cells and this may explain the N-fixation we observed in the autoclaved soils. In addition, the use of 300 μg NH_4NO_3 g^{-1} soil may have been insufficient N to completely inhibit N-fixation as amounts of 300 mg NH_4NO_3 g^{-1} soil are more commonly used in inhibition experiments with soil. A second experiment was conducted to further explore these variables and to extend our observations to a second soil type.

Experiment 2: Caldwell Field Soil

The experimental procedures were performed as described above except that soils were obtained from a new site, Caldwell field G sampled on 07/06/2007, and the treatments were modified. Treatments were as follows:

Water only: 500 μl of sterile water was added to 5 g soil

Glucose: 500 μl of glucose (25% w/v) was added into 5 g of soil (25 mg glucose g^{-1} soil final).

Nitrogen: 500 μl of NH_4NO_3 (250 mg/ml) added into 5g of soil (25 mg NH_4NO_3 g^{-1} soil final).

Glucose + Nitrogen: Glucose and nitrogen were added as described above.

Glucose + Acetylene: glucose was added as described above and 40% acetylene was added to the headspace (40% N_2, 40% C_2H_4 and 20% O_2).

Autoclaved + Glucose: Soil samples (5 g) were autoclaved as a thin layer in a tin dish for 6 of the following cycles. Each cycle consisted of 2 h in the autoclave followed by addition of 1 ml sterile water and then incubation at 30°C overnight.

Autoclaved + Glucose + Nitrogen: Soil was autoclaved and glucose and nitrogen were added as described above and

Caldwell field soils were observed to have a much lower N-fixation rate than the Chazy T2 soils and this result was significant (Mann Whitney U-test, $P < 0.05$). Glucose significantly increased the N-fixation rate relative to water only control but no change in N-fixation rate was observed when glucose was added with excess nitrogen or with excess acetylene (Table 2). Acetylene binds competitively with N_2 at the active site of nitrogenase and when in excess should inhibit N-fixation. The

autoclave treatment nearly eliminated the stimulatory effect of glucose (Table 2). However, N-fixation was still observed in autoclaved soils where glucose was added. Addition of nitrogen and glucose to the autoclaved soil reduced N-fixation significantly relative to autoclaved soil that received glucose alone indicating that this is a biological response (Table 2). The result suggests that the autoclave treatment was still not sufficient to completely sterilize the soil.

Table 2. N-fixation rates from Caldwell soil, Experiment 2

Treatment	*N-fixed* *(ug kg^{-1} d^{-1})*
Water only	1.4 ± 0.3[2, 3]
Glucose	4144 ± 179[1, 3]
Nitrogen	1.19 ± 0.9[2, 3]
Glucose + Nitrogen	1.19 ± 0.6[2, 3]
Glucose + Acetylene	0.5 ± 0.1[1, 2, 3]
Autoclaved + Glucose	6.9 ± 0.7[1, 2]
Autoclaved + Glucose + Nitrogen	5.4 ± 1.1[1, 2, 3]

[1] Values different from 'Water only' by U-Test at $P < 0.05$.
[2] Values different from 'Glucose' by U-Test at $P < 0.05$.
[3] Values different from 'Autoclaved + Glucose' by U-Test at $P < 0.05$.

An unexpected result is that autoclaved soils that received glucose plus nitrogen had higher rates of N-fixation than did soils that only received glucose plus nitrogen and were not autoclaved. One explanation is that the organisms that survived autoclave treatment belonged to specific group of N-fixers (e.g.: spore forming organisms) and that the members of this surviving group are for some reason less sensitive to N fertilization than the majority community. Another explanation would be that autoclaving released nutrients or increased the bioavailability of trace elements which were then available to the organisms that survived autoclaving. The result cannot be explained by variation due to denitrification or other sources of gaseous N-losses. The $\delta^{15}N$ of autoclaved + glucose + nitrogen control soils (those receiving

unlabeled N_2) was 0.39 ± 0.02 while that from glucose + nitrogen control soils was 0.44 ± 0.05; a difference that is not significant and not able to describe the ^{15}N enrichment observed in autoclaved + glucose + nitrogen soils that received $^{15}N_2$. Thus, $^{15}N_2$ fixation is the only process that can explain the ^{15}N signature of the autoclaved + glucose + nitrogen soils that received $^{15}N_2$.

The assay we describe measures specific incorporation of ^{15}N from $^{15}N_2$ into the total soil N pool. There are no chemical, physical, or biological processes other than N-fixation that are known to can carry out this reaction at low temperature and pressure. Fixation followed by mineralization would have no impact on the measurement since the experimental design prevents leaching losses and all mineral N-is included in the analysis of the total soil N pool. Gaseous losses of N from soil such as those mediated by nitrification or denitrification could impact the ^{15}N-signature of the total soil N pool as a result of fractionation or if these processes target N pools with a ^{15}N signature that deviates significantly from the bulk soil. However, these processes are accounted for by determining ^{15}N enrichment in samples receiving $^{15}N_2$ relative to those receiving unenriched N_2. The approach we describe could underestimate N-fixation if N-fixation is tightly coupled to N- mineralization and N-mineralization is tightly coupled to denitrification. This result seems unlikely in situ since nitrification is favored when mineral N is low while the later processes are favored when mineral N is high.

The two different soils tested were observed to have very different potential N-fixation rates. For perspective, the rates observed would correspond to approximately 0.26 and 9 x 10^{-4} kg ha^{-1} d^{-1} N-fixed in the Chazy and Caldwell sites respectively,

which amount to 55 and 0.2 kg ha^{-1} d^{-1} N-fixed from May through November. In both soils the addition of glucose dramatically stimulated N-fixation. Addition of mineral N decreased N-fixation in both soils. The first experiment involved a low level mineral N amendment resulting in partial inhibition of N-fixation. A larger amount of mineral N was added in the second experiment, but while the rate of N-fixation was reduced slightly in response to N amendment, the low N-fixation rate made it difficult to evaluate whether the rate reduction was statistically significant. The ability of glucose to stimulate N-fixation in both soils with low and high fixation rate, and the nearly complete inhibition of this stimulatory effect in the Caldwell soil by autoclaving or the addition of mineral N, or acetylene, confirms that the assay we are using is specific for N-fixation.